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(54) Title: CONGENIC ANIMAL MODELS OF NON-INSULIN DEPENDENT DIABETES MELLITUS

(57) Abstract: Congenic animals and animal populations having type II diabetes-associated phenotypes are described. Insulin degradation polypeptides having amino acid substitutions linked to a type II diabetes-associated phenotypes also are described.

CONGENIC ANIMAL MODELS OF NON-INSULIN DEPENDENT DIABETES MELLITUS

TECHNICAL FIELD

The invention relates to non-human congenic animals and congenic animal populations that exhibit a type II diabetes-associated phenotype, as well as insulin degradation polypeptides having substitutions that confer type II diabetes-associated phenotypes.

BACKGROUND OF THE INVENTION

Type II diabetes or non-insulin dependent diabetes mellitus (NIDDM) is an increasing health burden in urbanized societies with aging populations, as the disease is associated with older, physically inactive, overweight individuals. Approximately 135 million people worldwide are affected and therefore are at an increased risk for myocardial infarction, stroke, end-stage kidney disease, vision defects, and neurological problems.

In general, it is considered that the disease results from a combination of impaired insulin action in target tissues and a reduced capacity to secrete insulin from the pancreatic 9-cells. Numerous family and twin studies have demonstrated the critical influence of environmental factors as well as a sizable impact of genetic factors for the risk to type II diabetes. Monogenic variants of diabetes with autosomal dominant mode of inheritance (MODY) or mitochondrial inheritance of disease have been described in recent years at the molecular and clinical levels. The common forms of the disease appear, however, to be multifactorial with influence of both polygenic and environmental factors.

SUMMARY OF THE INVENTION

The invention is based on the development of congenic animals and congenic animal populations that have a type II diabetes-associated phenotype. Development of congenic animal strains allows susceptibility genes residing within quantitative trait loci (QTLs) to be identified,

as well as the pathophysiological implications of such genes to be characterized. As the congenic animals of the invention have a type II diabetes-associated phenotype, genetic fine mapping also can be performed, so that associated genes, such as the variant of an insulin degradation enzyme described herein, can be positionally cloned. Furthermore, physiological characterization of congenic strains and heterozygous backcross animals provides clues to the contribution of a single QTL to the pathophysiology of a complex phenotype. *Niddm1* congenic strains of the invention provide specific animal models for mild type II diabetes that will allow pathophysiological mechanisms of the disease to be refined, and provide a tool for screening pharmaceutical agents.

In one aspect, the invention features a non-human congenic animal that includes genetic material of a donor animal and a recipient animal. The congenic animal exhibits a type II diabetes-associated phenotype, wherein less than about one chromosome (e.g., less than about 50 cM, 20 cM, 10 cM, or 5 cM) of the congenic animal's genome is derived from the donor animal, and wherein the genetic material from the donor is necessary for expression of the type II diabetes-associated phenotype in the congenic animal. The congenic animal can be marker-defined. Substantially all mitochondria of the congenic animal can be derived from either the recipient animal or the donor animal. The type II diabetes-associated phenotype can be selected from the group consisting of elevated postprandial glycemia, hypertension, glucose intolerance, insulin resistance, abnormal insulin secretion, reduced insulin action, increased body weight, dyslipidemia, hyperinsulinemia, impaired lipogenesis, altered glycogen metabolism, altered coagulation atherosclerosis, altered kidney function, altered nerve function, altered eye function, obesity, and inflammation.

The donor animal's genome can include a *Niddm1a* genomic interval. The congenic animal's genome derived from the donor can include a genomic interval selected from the group consisting of *Niddm1a, Niddm1b, Niddm1c, Niddm1d, Niddm1e, Niddm1f, Niddm1g, Niddm1h,* and *Niddm1i.* For example, the genomic interval can be a *Niddm1e* genomic interval. The congenic animal's genome derived from the donor also can be selected from a genomic interval selected from the group consisting of *NiddmC2, NiddmC3, NiddmC5, NiddmC7, NiddmC9A, NiddmC9B, NiddmC10, NiddmC11, NiddmC13, NiddmC18, NiddmC(13+15),* and *NiddmC(9+13+15).*

The invention also features an isolated cell of a congenic animal of the invention as well as a tissue culture derived from a congenic animal of the invention. The cell can be selected from the group consisting of adipocytes, mesangial cells, hepatic cells, pancreatic cells, muscle cells, endothelial cells, and neural cells. The tissue culture can be selected from the group consisting of adipose tissue, mesangial tissue, hepatic tissue, pancreatic tissue, muscle tissue, blood-vessel tissue, and neural tissue.

Congenic animals of the invention can be non-human mammals (e.g., a rodent such as a rat, mouse, or guinea pig, or a swine), insects, or birds. The rodent can be a rat.

The invention also features non-human congenic animal obtained by crossing a first non-human congenic animal with a second non-human congenic animal, wherein the first and second congenic animals have type II diabetes-associated phenotypes. The first and second congenic animals can have distinct metabolic phenotypes and/or have non-overlapping genomic intervals. Such congenic animals are effective for evaluating epistatic interactions between the non-overlapping genomic intervals.

In another aspect, the invention features a non-human congenic animal population that includes a plurality of non-human congenic animals. The congenic animals exhibit a plurality of type II diabetes-associated phenotypes, wherein each congenic animal within the plurality of congenic animals includes genetic material from a donor animal and a recipient animal, wherein about 0.1% to about 50% of each congenic animal's genome is derived from the donor animal, and wherein the genetic material from the donor is necessary for expression of the type II diabetes-associated phenotype in each congenic animal.

The invention also features a method for testing a pharmaceutically active compound. The method includes administering a test compound to a non-human congenic animal exhibiting a type II diabetes-associated phenotype, wherein the non-human congenic animal includes

genetic material of a donor animal and a recipient animal, wherein less than about 50 cM of the congenic animal's genome is derived from the donor animal, and wherein the genetic material from the donor is necessary for expression of the type II diabetes-associated phenotype in the congenic animal; and evaluating the test compound for an effect on at least one type II diabetes-associated phenotype in the congenic animal. The congenic animal can include the genetic intervals as described above. The animal can include a progeny animal of a cross between two congenic parent animals, the parent animals having distinct congenic intervals.

In another aspect, the invention features a method for testing a pharmaceutically active compound. The method includes administering a test compound to a plurality of non-human congenic animals exhibiting a plurality of type II diabetes-associated phenotypes; and evaluating the test compound for an effect on at least one type II diabetes-associated phenotype, wherein each congenic animal within the plurality of congenic animals includes genetic material from a donor animal and a recipient animal, wherein about 0.1% to about 50% of each congenic animal's genome is derived from the donor animal, and wherein the genetic material from the donor is necessary for expression of the type II diabetes-associated phenotype in each congenic animal. The plurality of congenic animals can include at least two rats having congenic intervals on different chromosomes.

The invention also features an article of manufacture that includes isolated cells of a non-human congenic animal exhibiting a type II diabetes-associated phenotype. The article further can include a label or package insert indicating the cells are useful for evaluating compounds that may be effective for alleviating type II diabetes-associated phenotypes.

In another aspect, the invention features a method of doing business. The method includes offering for sale a non-human congenic animal exhibiting a type II diabetes-associated phenotype, or a cell derived therefrom; and communicating that the animal is effective for testing or evaluating compounds that are effective for alleviating type II diabetes-associated phenotypes.

In yet another aspect, the invention features a method of making a non-human congenic animal that includes mating a donor animal and a recipient animal to produce a progeny animal; and successively backcrossing the progeny animal with the recipient animal for at least 10 generations to produce the congenic animal, wherein the congenic animal exhibiting a type II diabetes-associated phenotype, wherein less than about 50 cM of the congenic animal's genome is derived from the donor animal, and wherein the genetic material of the donor is necessary for expression of the type II diabetes-associated phenotype in the congenic animal.

The invention also features an isolated insulin degradation polypeptide and an isolated polynucleotide encoding the insulin degradation polypeptide, wherein the polypeptide includes at least one amino acid substitution, wherein the amino acid substitution is linked to a type II diabetes-associated phenotype. The polypeptide can include at least one amino acid substitution in the amino acid sequence of SEQ ID NO:23, e.g., an arginine residue substituted at amino acid 18 and/or a valine residue is substituted at amino acid 890 of SEQ ID NO:23. The polynucleotide can have a cytosine residue at nucleotide 2817 of SEQ ID NO:22.

The invention also features a transgenic non-human animal whose genome includes an insulin-degrading polypeptide transgene, wherein the transgene includes a regulatory polynucleotide operably linked to a polynucleotide encoding an insulin-degrading polypeptide, wherein the insulin-degrading polypeptide has an amino acid substitution linked to a type II diabetes-associated phenotype. The animal can be a rat or a mouse.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure1 is a genetic map of the distal part of rat chromosome 1 in congenic strains *Niddm1a*, *Niddm1b*, and *Niddm1i*. The extents of Goto-Kakizaki (GK) derived genomic intervals are displayed as black bars for the three congenic strains. White bars indicate genomic intervals spanning the crossover points between GK and F344 derived alleles, as defined by the closest flanking markers.

Figures 2A-2D are graphs that depict intraperitoneal glucose tolerance test results of *Niddm1* congenics and F344 rats. Male rats (95 days) from strains *Niddm1a* (n = 11), *Niddm1b* (n = 17), *Niddm1i* (n = 12), and F344 (n = 20) were subjected to IPGTT. After glucose injection, the concentrations of blood glucose (2A, 2B) and serum insulin (2C, 2D) were determined at the indicated time points. Results are shown as mean±sem.

Figures 3A-3D are graphs that depict glucose incorporation into lipids as a result of insulin stimulated synthesis in F344, GK, *Niddm1b*, and *Niddm1i* rats. Adipocytes were isolated from epididymal fat of two month old male F344 (n = 7), GK (n = 4), *Niddm1b* (n = 5), and *Niddm1i* (n = 5) rats, and incubated for 2 h with insulin (0-20,000 μU/ml). Figure 3A indicates that glucose incorporation into lipids (lipogenesis) in the absence of insulin (basal conditions) was higher in F344 rats than GK ($p = 0.009$), *Niddm1b* ($p = 0.007$), and *Niddm1i* ($p = 0.04$) rats. Figure 3B indicates maximal insulin induced lipogenesis was higher in F344 than in GK ($p = 0.00004$), *Niddm1b* ($p = 0.008$), and *Niddm1i* ($p = 0.001$) rats. Maximal insulin induced lipogenesis was higher in *Niddm1b* and *Niddm1i* rats compared with GK ($p = 0.02$ and 0.006) rats. Figures 3C and 3D indicate dose dependent insulin stimulated lipogenesis expressed as an increase above values (mean∀sem) obtained without insulin (3C) or in percent of maximum (3D).

Figure 4 is a graph that depicts quantitative analysis of insulin RNA in GK, F344, and *Niddm1* rats. Results are shown as mean ∀ sem. The amounts of RNA are expressed as pixels, and were calculated from band intensities using phosphorimaging techniques.

Figure 5 is a genetic map of part of rat chromosome 1 in congenic rat strains *Niddm1b*, *Niddm1c*, *Niddmf*, and *Niddm1e*. The extents of GK derived genomic intervals are displayed as black bars for the four congenic strains. White bars indicate genomic intervals spanning the crossover points between GK and F344 derived alleles, as defined by the closest flanking markers.

Figures 6A-6B are graphs that depict lipogenesis in adipocytes isolated from epididymal fat. Adipocytes were isolated from two-month old male F344 (n=6), *Niddm1f* (n = 5), and *Niddm1e* (n = 4) rats, and incubated for 2 h with insulin (0-20,000 TU/ml). Figure 6A indicates that lipogenesis in the absence of insulin (basal conditions) was higher in F344 than *Niddm1f* (p=0.001) and *Niddm1e* (p=0.002) rats. Figure 6B indicates maximal insulin induced lipogenesis was higher in F344 than in *Niddm1f* (p=0.00001) and *Niddm1e* (p=0.003) rats.

Figure 7 is a schematic of the translated part of the gene encoding a rat insulin degradation enzyme (IDE).

Figure 8 is a graph that depicts insulin degrading activities of wild-type IDE and IDE variants A890V (i.e, valine for alanine at amino acid 890), H18R (i.e, arginine for histidine at amino acid 18), and A890V+H18R in intact COS-1 cells. All values are from four separate transfections and are expressed as the percentage of wild type activity (cells transfected by pCMV4-Ide from F344 rat), which is arbitrarily defined as 100%. Within each experiment, the background COS-1 insulin degrading activity was subtracted from each individual value and activities were corrected for both the total protein content and ϑ-galactosidase activity. Actual

values (mean∀sem) for A890V, H18R, and A890R+H18R are 95∀9, 89∀8, and 69∀6%, respectively.

DETAILED DESCRIPTION

Congenic Animal Models of Type II Diabetes

The invention features non-human congenic animals identified following mating of a donor and a recipient animal, and cells and tissues derived from such congenic animals. In general, congenic animals contain a discrete portion of genetic material (genomic interval) from one animal strain (i.e., the donor) in the context of the genetic background of a second animal strain (i.e., the recipient). Non-human animals that are capable of being inbred are suitable for use as donor and recipient animals. Non-limiting examples include rodents such as mice, rats, rabbits, and guinea pigs, pigs, cattle, goats, fish, and birds such as turkeys and chickens. Rats, mice, and pigs are particularly useful animals. Typically, "donor" refers to an animal that has a genetically linked, type II diabetes-associated phenotype. Donor animals can be, for example, GK rats, Long-Evans Tokushima Fatty (OLETF) rats, NZO mice, and NON mice. See, for example, Kim et al., Physiol. Pharmacol., 1998, 9(2-4):325-345.

The GK rat is an extensively studied animal model for type II diabetes. The phenotype of GK animals is well characterized and includes several features typical of type II diabetes, such as fasting hyperglycemia, impaired secretion of insulin in response to glucose, and insulin resistance, as well as late complications, e.g., neuropathy and nephropathy. Genetic linkage analysis of an F2-intercross between a GK and a normoglycemic F344 rat identified four major QTL with genome-wide significance (*Niddm1*, *Niddm2*, *Niddm3*, and *weight1*), as well as 10 minor QTLs that affect the segregation of diabetes and its associated phenotypes. Galli, J. et al., Nature Genet., 1996, 12:31-37. OLETF rats exhibit mild obesity and develop gender dimorphic NIDDM with aging. Analysis of an F_2-intercross between OLETF and BN or F rats identified *Dmo1* as a QTL associated with glucose intolerance, fasting plasma glucose levels, and body weight, and is found in the *Niddm1* region of rat chromosome 1. Kim et al., 1998, *supra*. Typically, "recipient" refers to an inbred animal that does not exhibit a type II diabetes-associated phenotype. Recipient animals can be, for example, Fischer-344, DA, LEW, ACI, WKY, SD, or BN rats, or BALB/c, FVB, or SSL mice.

Generally, the donor animal exhibits a type II diabetes-associated phenotype while the recipient animal does not. Mating of such animals allows the type II diabetes-associated alleles to be introgressed into the context of the non-type II diabetes-associated phenotype. Alternatively, the recipient animal exhibits a type II diabetes-associated phenotype while the donor animal does not. Mating of such animals allows non-type II diabetes-associated alleles to be introgressed into the context of a type II diabetes-associated phenotype.

After mating of a donor and a recipient, progeny are successively backcrossed with recipient animals to introgress alleles of interest onto the genome of the recipient to produce congenic animals. Typically, the congenic animals are identified from at least an F10 generation. Alternatively, a procedure referred to as "speed congenics" or "marker-assisted breeding" can be used. See, for example, Whittaker et al., Genet. Res., 66(3):255-265, 1995; and Darvasi, Nat. Genet., 18(1):19-24, 1998. In this method, progeny in each backcross generation are chosen that have lost the maximum number of donor background alleles. Less breeding is required in this method, such that congenic animals can be identified earlier than the F10 generation (e.g., F9 generation). The phenotype of progeny can be assessed at each generation by, for example, an intraperitoneal or intravenous glucose tolerance test, in which serum glucose and insulin levels are determined in fasted animals that have been injected with glucose. In addition, insulin tolerance tests, in which the glucose levels are determined in animals after injection of insulin or tests in which nutrient or hormone levels are determined following fasting and/or provocation can be used to phenotype the animals. Substantially all the mitochondria of the congenic animals can be derived from either the donor or the recipient, as mitochondria are maternally inherited.

Genotype can be assessed in congenic animals of the invention using known genetic markers. For example, the presence of microsatellites or simple sequence length polymorphisms (SSLPs), composed of mono-, di-, tri-, or tetrameric sequences repeated multiple times in a tandem array, can be assessed by amplification of the region surrounding a microsatellite or SSLP using the polymerase chain reaction (PCR). In some embodiments, congenic animals of the invention may be characterized as "marker-defined", which indicates the animals are

genetically pure when genotyped as described above. Thus, if the donor animal was a GK rat, a marker-defined congenic animal would have all markers from the recipient animal, except for a GK-specific region, which typically is less than one chromosome in length.

Thus, a major QTL such as *Niddm1*, which explains approximately 30% of the genetic effects on postprandial glucose concentrations, can be sorted into discrete genetic factors by establishing congenic strains covering different parts of the QTL. For example, congenic strains can be established by transferring *Niddm1*-GK alleles onto the genome of the normoglycemic F344 rat. The region can be less than, for example, about 50 centimorgans (cM), 20 cM, 10 cM, or 5 cM in length. As described herein, the *Niddm1a*, *Niddm1b*, *Niddm1i*, *Niddm1e*, *Niddm1d*, *Niddm1f*, *Niddm1g*, and *Niddm1h* congenic strains have about 52, 28, 22, 3, 19, 8, 13, and 24 cM derived from the genome of GK rats, respectively. The *Niddm1* locus was dissected into two genetic entities defined by the non-overlapping congenic strains *Niddm1b* and *Niddm1i*, with each genetic entity having distinct effects on the diabetic phenotype. Congenic animals of the invention exhibit a type II diabetes-associated phenotype including one or more of the following: elevated postprandial hyperglycemia, hypertension, glucose intolerance, insulin resistance, altered insulin secretion, reduced insulin action, increased body weight, dyslipidemia, hyperinsulinemia, impaired lipogenesis, altered glycogen metabolism, altered coagulation, atherosclerosis, altered kidney function (e.g., nephropathy), altered eye function (e.g., retinopathy), altered nerve function (e.g. neurapathy), and macro- or microangiopathy. For example, congenic strains *Niddm1b* and *Niddm1i* each displayed elevated postprandial glucose levels and impaired basal and insulin induced lipogenesis in isolated adipocytes *in vitro*. Several features, however, are unique to the respective strains. *Niddm1i* rats display insulin resistance in combination with a severe reduction of insulin secretion *in vivo*. This sub-strain of the *Niddm1* QTL did not develop increased body weight, epididymal fat mass, or increased levels of triglycerides. Thus, the phenotype is similar to that of patients with MODY with early defects in insulin secretion. The mode of inheritance, however, is apparently recessive since the insulin secretion defect was not observed in *Niddm1i*/F344 heterozygous rats. Insulin levels during IPGTT were reduced in young *Niddm1i* rats, although the postprandial glucose levels were barely higher than in F344, possibly indicating an important contribution of insulin independent glucose disposal at this age.

In diabetes patients, as well as in GK rats, defects in both insulin secretion and insulin action are implicated in the development of the disease. The relative etiological importance of these defects is still controversial. Since postprandial glucose levels are only slightly elevated and basal glucose is normal in 65 day old *Niddm1i* rats, the defects in insulin secretion and action are most likely not merely consequences of glucotoxicity. Without being bound by a particular mechanism, *Niddm1i* may have impaired mechanisms common to insulin secretion in pancreatic 9-cells and insulin action in adipocytes. Similar to the gene encoding insulin receptor substrate 2, IRS-2, which causes defects in both insulin secretion and action in mice. Withers, D.J. et al., Nature, 1998, 391:900-904. The *Irs-2* gene is located on chromosome 13 in humans and chromosome 8 in mice. According to syntenic conservation, the *Irs-2* gene is not a candidate gene for the *Niddm1i* phenotype.

Young *Niddm1b* and heterozygous *Niddm1b*/F344 rats have slightly elevated postprandial glucose levels, but substantially elevated insulin levels, indicating that insulin resistance is compensated by increased insulin secretion. In older heterozygous rats, impaired insulin action can still be compensated but not in the homozygous *Niddm1b* rats that develop fasting hyperglycemia, fasting hyperinsulinemia, increased body weight and epididymal fat mass, as well as dyslipidemia. This constellation is well recognized in diabetes patients, in whom insulin resistance is considered as a cornerstone in the metabolic syndrome. That insulin resistance in *Niddm1b* rats is a likely primary defect is supported by the fact that *Niddm1b*/F344 heterozygous rats also exhibit signs of insulin resistance, but display normal or below normal levels of the other diabetes-associated phenotypes.

Insulin resistance and diabetes in humans are often associated with hypertriglyceridemia, increased levels of LDL cholesterol, and decreased levels of HDL cholesterol. *Niddm1b* rats exhibit increased triglyceride levels in combination with increased total cholesterol and HDL cholesterol. The difference between total cholesterol and HDL cholesterol should approximately reflect the LDL and VLDL cholesterol levels, for which no difference was observed between *Niddm1b* and F344 rats. Thus, the disordered lipid metabolism in *Niddm1b* does not exactly fit the pattern in diabetes patients. This discrepancy probably reflects a species-specific difference in the manifestation of dyslipidemia in rodents as compared with humans.

Data described herein indicate the presence of non-allelic interaction or epistasis between the two diabetes loci *Niddm1b* and *Niddm1i*. In *Niddm1a* (encompassing both *Niddm1b* and *Niddm1i*), as compared with F344 rats, the elevation of postprandial glucose levels was less severe than might be expected from the additive effect of the two sub-strains. Interpreting the epistasis in physiological terms suggests that counter-regulatory mechanisms that protect the organism against excessive glucose concentrations, restrict the hyperglycemia, unless the animals are carrying additional diabetes genes (as in GK) or are subjected to environmental stress.

The homologous chromosomal regions corresponding to *Niddm1a* in humans are 11q13, 9p24, and 10q24-26. Interestingly, a locus that was linked to diabetes in a Mexican-American population was recently reported on chromosome 10q. The authors also reported a locus with suggestive linkage to diabetes on human chromosome 9p, which corresponds to *Niddm1b*.

Additional congenic animals of the invention can be produced by crossing a first congenic animal with one or more second congenic animals. The first and second congenic animals each may be obtained from a F10 generation following a mating of a donor and recipient animal, as described above. Typically, the first and second congenic animals have non-overlapping genomic intervals derived from the donor, and typically, distinct type II diabetes-associated phenotypes. Congenic animals obtained from such crosses are effective for evaluating epistatic interactions between non-overlapping intervals.

Congenic Animal Populations

The invention features congenic animal populations that exhibit a plurality of type II diabetes-associated phenotypes. Congenic animal populations are identified from a mating of a donor and a recipient animal, as described above, but contain a plurality of animals from the F3 generation through at least the F10 generation (e.g., F12 generation). Each animal in such an animal population has from about 0.1% to about 50% of its genome derived from the donor animal. Thus, each animal in the congenic animal population has a discrete portion of its

genome, which is distinct from other congenic animals in the population, derived from the donor animal.

Congenic animal populations of the invention and tissues, cells, and cellular extracts derived therefrom, are effective for evaluating epistatic effects of type II diabetes-associated phenotypes and can be used to identify pharmaceutical agents that may be useful for treating type II diabetes. For example, a test compound is administered to a congenic animal or congenic animal population of the invention, and a diabetes-associated phenotype, such as elevated postprandial hyperglycemia, hypertension, glucose intolerance, insulin resistance, altered insulin secretion, reduced insulin action, increased body weight, dyslipidemia, hyperinsulinemia, impaired lipogenesis, and altered glycogen metabolism is monitored in relation to control animals. Test compounds can be formulated into pharmaceutical compositions by admixture with pharmaceutically acceptable non-toxic excipients or carriers and administered to congenic animals of the invention by any route of administration. For example, parenteral routes such as subcutaneous, intramuscular, intravascular, intradermal, intranasal, inhalation, intrathecal, or intraperitoneal administration, and enteral routes such as sublingual, oral, or rectal administration can be used.

Insulin Degrading Polypeptides

The invention features isolated, insulin degrading enzyme (IDE) polypeptides that contain at least one amino acid substitution compared to the native polypeptide, linked to a type II diabetes-associated phenotype. As used herein, the term "polypeptide" is any chain of amino acids, regardless of length or post-translational modification. Amino acids have been designated herein by standard three letter and one letter abbreviations. Agents (e.g., small molecules or biological macromolecules) that affect IDE activity or expression can be identified with standard methodologies.

IDE is a metalloprotease that binds insulin with high specificity and a low Km, and plays an important role in cellular processing and degradation of insulin. IDE requires Zn^{2+} for activity, but does not contain a typical Zn^{2+} binding site, and belongs to a new class of proteases

that contains an HXXEH active-site motif. The IDE protein is evolutionary well conserved, indicating that in addition to its insulin clearing function, it probably has other, more complex, cellular functions. IDE is localized in several cellular compartments including the cell surface, endosomes, cytoplasm, and peroxysomes, and is widely expressed in the body. Although insulin is the substrate with the greatest affinity for IDE, the protein interacts with several other growth factors, such as proinsulin, epidermal growth factor, and insulin-like growth factor-1 (IGF-1), that are bound but poorly degraded by IDE, and IGF-II, atrial natriuretic peptide, and transforming growth factor-I, that bind to IDE and are readily degraded. Studies also have implicated a role of IDE in other types of cellular proteolysis, as IDE has been shown to interact with the proteasome, a major site for intra-cellular protein degradation. Another implicated function for IDE is a regulatory role in steroid action, since it has been shown that IDE interacts with both the glucocorticoid and androgen receptor. See, for example, Authier et al., Clin. Invest. Med., 1996, 19(3):149-160.

Modifications of the insulin-degrading polypeptide can include, for example, at least one amino acid substitution at residue 18 or 890 of the amino acid sequence of SEQ ID NO:23. The substitutions may be conservative or non-conservative. Conservative amino acid substitutions replace an amino acid with an amino acid of the same class, whereas non-conservative amino acid substitutions replace an amino acid with an amino acid of a different class. Examples of conservative substitutions include an arginine for a histidine at residue 18 (H18R) and a valine for an alanine at residue 890 (A890V) of SEQ ID NO:23. Non-conservative substitutions may result in a substantial change in the hydrophobicity of the polypeptide or in the bulk of a residue side chain. In addition, non-conservative substitutions may make a substantial change in the charge of the polypeptide, such as reducing electropositive charges or introducing electronegative charges. Examples of non-conservative substitutions include a basic amino acid for a non-polar amino acid, or a polar amino acid for an acidic amino acid.

As described herein, the *Niddm1b* locus was sub-mapped to a small genetic region of approximately 3.7 cM defined by congenic strain *Niddm1e*. The gene encoding IDE was mapped within this region, and a GK-specific allelic variant of IDE was identified. Two nucleotide variations in the translated region of the GK allele resulted in the amino-acid changes H18R and A890V. The IDE cDNA was sequenced in 12 other rat strains to investigate the

frequency of the identified variants. A890V was unique for GK while the H18R was present in approximately 50% of the analyzed rat strains, indicating that the A890V variant could be of importance for the diabetic phenotype. Furthermore, *in vitro* expression analysis showed about a 30% reduction in insulin degradation by the GK variant containing both changes. When H18R and A890V variants were studied separately, no significant effect was observed for A890V, and H18R showed only a slightly reduced insulin degrading capacity. This indicates that the two variants are interacting synergistically to mediate the effect on insulin degradation. As the GK variants had no impact on insulin degradation in cell lysates of Ide transfected cells, the defect in IDE is specific and likely coupled to receptor-mediated internalization of insulin. It is noteworthy that the real effect of the Ide GK variant could even be larger than detected, since it is known that up to 50% of the insulin is degraded by IDE directly on the surface of cultured cells.

Nucleic Acids encoding modified insulin-degrading polypeptides

Isolated nucleic acid molecules encoding modified insulin-degrading polypeptides of the invention can be produced by standard techniques. As used herein, "isolated" refers to a sequence corresponding to part or all of a gene encoding a modified insulin-degrading polypeptide, but free of sequences that normally flank one or both sides of the wild-type gene in a mammalian genome. An isolated polynucleotide can be, for example, a recombinant DNA molecule, provided one or both of the nucleic acid sequences normally found immediately flanking that recombinant DNA molecule in a naturally-occurring genome is removed or absent. Thus, isolated polynucleotides include, without limitation, a recombinant DNA that exists as a separate molecule (e.g., a cDNA or genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences as well as recombinant DNA that is incorporated into a vector, an autonomously replicating plasmid, a virus (e.g., a retrovirus, adenovirus, or herpes virus), or into the genomic DNA of a prokaryote or eukaryote. In addition, an isolated polynucleotide can include a recombinant DNA molecule that is part of a hybrid or fusion polynucleotide.

It will be apparent to those of skill in the art that a polynucleotide existing among hundreds to millions of other polynucleotides within, for example, cDNA or genomic libraries,

or gel slices containing a genomic DNA restriction digest is not to be considered an isolated polynucleotide.

Isolated polynucleotides are at least about 14 nucleotides in length and contain a substitution in the sequence from the wild-type. For example, the nucleic acid can contain a guanine at nucleotide 68, a thymine at nucleotide 2684, or a cytosine at nucleotide 2817 of SEQ ID NO:22. The nucleic acid molecule can be about 14 to 20, 20-50, 50-100, or greater than 150 nucleotides in length. In some embodiments, the isolated nucleic acid molecules encode a full-length, modified insulin-degrading polypeptide. Nucleic acid molecules can be DNA or RNA, linear or circular, and in sense or antisense orientation.

Specific point changes can be introduced into the nucleic acid molecule encoding wild-type insulin-degrading polypeptides by, for example, oligonucleotide-directed mutagenesis. In this method, a desired change is incorporated into an oligonucleotide, which then is hybridized to the wild-type nucleic acid. The oligonucleotide is extended with a DNA polymerase, creating a heteroduplex that contains a mismatch at the introduced point change, and a single-stranded nick at the 5' end, which is sealed by a DNA ligase. The mismatch is repaired upon transformation of *E. coli*, and the gene encoding the modified insulin-degrading polypeptide can be re-isolated from *E. coli*. Kits for introducing site-directed mutations can be purchased commercially. For example, Muta-Gene7 *in-vitro* mutagenesis kits can be purchased from Bio-Rad Laboratories, Inc. (Hercules, CA).

PCR techniques also can be used to introduce mutations. See, for example, Vallette et al., Nucleic Acids Res., 1989, 17(2):723-733. PCR refers to a procedure or technique in which target nucleic acids are amplified. Sequence information from the ends of the region of interest or beyond typically is employed to design oligonucleotide primers that are identical in sequence to opposite strands of the template to be amplified, whereas for introduction of mutations, oligonucleotides that incorporate the desired change are used to amplify the nucleic acid sequence of interest. PCR can be used to amplify specific sequences from DNA as well as RNA, including sequences from total genomic DNA or total cellular RNA. Primers are typically 14 to 40 nucleotides in length, but can range from 10 nucleotides to hundreds of nucleotides in length.

General PCR techniques are described, for example, in PCR Primer: A Laboratory Manual, Ed. by Dieffenbach, C. and Dveksler, G., Cold Spring Harbor Laboratory Press, 1995.

Nucleic acids encoding modified insulin-degrading polypeptides also can be produced by chemical synthesis, either as a single nucleic acid molecule or as a series of oligonucleotides. For example, one or more pairs of long oligonucleotides (e.g., >100 nucleotides) can be synthesized that contain the desired sequence, with each pair containing a short segment of complementarity (e.g., about 15 nucleotides) such that a duplex is formed when the oligonucleotide pair is annealed. DNA polymerase is used to extend the oligonucleotides, resulting in a double-stranded nucleic acid molecule per oligonucleotide pair, which then can be ligated into a vector.

Production of modified insulin-degrading polypeptides

Modified insulin-degrading polypeptides of the invention can be produced by ligating a nucleic acid molecule encoding the polypeptide into a nucleic acid construct such as an expression vector, and transforming a bacterial or eukaryotic host cell with the expression vector. In general, nucleic acid constructs include a regulatory sequence operably linked to a nucleic acid sequence encoding an insulin-degrading polypeptide. Regulatory sequences do not typically encode a gene product, but instead affect the expression of the nucleic acid sequence. As used herein, "operably linked" refers to connection of the regulatory sequences to the nucleic acid sequence in such a way as to facilitate transcription and translation of the nucleic acid sequence. Regulatory elements can include, for example, promoter sequences, enhancer sequences, response elements, or inducible elements.

In bacterial systems, a strain of *Escherichia coli* such as BL-21 can be used. Suitable *E. coli* vectors include without limitation the pGEX series of vectors that produce fusion proteins with glutathione S-transferase (GST). Transformed *E. coli* are typically grown exponentially, then stimulated with isopropylthiogalactopyranoside (IPTG) prior to harvesting. In general, such fusion proteins are soluble and can be purified easily from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX

vectors are designed to include thrombin or factor Xa protease cleavage sites such that the cloned target gene product can be released from the GST moiety.

In eukaryotic host cells, a number of viral-based expression systems can be utilized to express modified insulin-degrading polypeptides. A nucleic acid encoding an insulin-degrading polypeptide can be cloned into, for example, a baculoviral vector such as pBlueBac (Invitrogen, San Diego, CA) and then used to co-transfect insect cells such as *Spodoptera frugiperda* (Sf9) cells with wild-type DNA from *Autographa californica* multiply enveloped nuclear polyhedrosis virus (AcMNPV). Recombinant viruses producing the modified insulin-degrading polypeptides can be identified by standard methodology. Alternatively, a nucleic acid encoding an insulin-degrading polypeptide can be introduced into a SV40, retroviral, or vaccinia based viral vector and used to infect host cells.

Mammalian cell lines that stably express modified insulin-degrading polypeptides can be produced by using expression vectors with the appropriate control elements and a selectable marker. For example, the eukaryotic expression vector pCDNA.3.1$^+$ (Invitrogen, San Diego, CA) is suitable for expression of modified insulin-degrading polypeptides in, for example, COS cells, HEK293 cells, or baby hamster kidney cells. Following introduction of the expression vector by electroporation, DEAE dextran-, calcium phosphate-, liposome-mediated transfection, or other suitable method, stable cell lines can be selected. Alternatively, transiently transfected cell lines are used to produce modified insulin-degrading polypeptides. Modified insulin-degrading polypeptides also can be transcribed and translated *in vitro* using wheat germ extract or rabbit reticulocyte lysate.

Modified insulin-degrading polypeptides can be purified by conventional chromatography methods or chemically synthesized using standard techniques. See, Muir, T.W. and Kent, S.B., Curr. Opin. Biotechnol., 1993, 4(4):420-427, for a review of protein synthesis techniques.

Transgenic Non-human Mammals

The invention also features a transgenic non-human mammal including a nucleic acid construct. As used herein, "transgenic non-human mammal" includes the founder transgenic non-human mammals as well as progeny of the founders. The nucleic acid construct includes a regulatory nucleic acid sequence operably linked to a polynucleotide encoding an insulin-degrading polypeptide, which contains at least one amino acid substitution linked to a type II diabetes-associated phenotype. Particularly useful substitutions are described above. Nucleic acid constructs can be produced through standard recombinant DNA techniques.

Transgenic non-human mammals can be farm animals such as pigs, goats, sheep, cows, horses, and rabbits, rodents such as rats, guinea pigs, and mice, and non-human primates such as baboons, monkeys, and chimpanzees. Transgenic mice are particularly useful.

Various techniques known in the art can be used to introduce nucleic acid constructs into non-human mammals to produce the founder lines of the transgenic non-human mammals. Such techniques include, but are not limited to, pronuclear microinjection (U.S Patent No. 4,873,191), retrovirus mediated gene transfer into germ lines (Van der Putten et al., Proc. Natl. Acad. Sci. USA, 82:6148, 1985), gene targeting into embryonic stem cells (Thompson et al., Cell, 56:313, 1989), electroporation of embryos (Lo, Mol. Cell. Biol., 3:1803, 1983), and transformation of somatic cells *in vitro* followed by nuclear transplantation (Wilmut et al., Nature, 385(6619):810-813, 1997; and Wakayama et al., Nature, 394:369-374, 1998).

Once transgenic non-human mammals have been generated, expression of the insulin-degrading polypeptide can be assessed using standard techniques. Initial screening can be accomplished by Southern blot analysis or PCR techniques to determine whether or not integration of the transgene has taken place. See, for example, sections 9.37-9.52 of Sambrook et al., 1989, *"Molecular Cloning, A Laboratory Manual"*, second edition, Cold Spring Harbor Press, Plainview; NY, for a description of Southern analysis.

Expression of the nucleic acid sequence encoding an insulin-degrading polypeptide in the tissues of the transgenic non-human mammals can be assessed using techniques that include, but are not limited to, Northern blot analysis of tissue samples obtained from the animal, *in situ* hybridization analysis, and reverse-transcriptase PCR (RT-PCR).

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1 - Materials and Methods: Inbred Fischer-344 (F344) rats were purchased from Charles River Laboratories and maintained by brother-sister mating. Rats had free access to tap water and chow and were maintained at a 12-h light and dark cycle (6am/6pm). Certain rats were fed a high fat diet containing 2% cholesterol, 20% olive oil, and 0.5% bile acid mixed in standard chow, starting at an age of 120 day.

Rat strain GK was obtained and bred as described by Galli et al., Nature Genet., 1996, 12:31-37. GK-derived genetic intervals were transferred onto the F344 genetic background by 10 successive backcrosses (F10) followed by intercrosses between heterozygous animals to establish homozygous congenic strains. At each generation, genetic markers from the *Niddm1* region were used to verify the integrity of the GK-susceptibility haplotype. The *Niddm1e*, *Niddm1f*, and *Niddm1c* congenic strains were bred by 12 successive backcrosses to F344 followed by intercrosses to establish homozygous lines.

Intraperitoneal glucose tolerance test: Intraperitoneal glucose tolerance tests (IPGTT) were performed on male rats of 95 and 225 days of age, as described by Galli et al., 1996, *supra*. Animals were fasted for 6-7 h; blood glucose levels were measured at 0 (baseline), 15, 30, 60, and 90 min after injection of 2.0 g glucose per kg body weight; and serum immunoreactive insulin levels were determined at 0, 15, and 30 min. Serum insulin levels in Tables 1, 2, and 3 were determined by an ELISA for rat insulin (Mercodia AB, Uppsala, Sweden), as described by

the manufacturer. Insulin values (Tg/l) obtained from the ELISA analysis were converted to pmol/l by multiplying by a factor of 174. The area under the curve (AUC) was calculated according to the trapezoid rule from glucose measurements at baseline, 15, 30, 60, and 90 min (mmol/l x min). Glucose and insulin values presented in Figure 2 were standardized by division with the corresponding mean values of F344 and subsequently multiplied by the mean values of F344 that are presented in Table 2. The glucose mean values of F344 in the experiment presented in Figure 2 are: 5.0 (basal), 18.6 (15 min), 13.8 (30 min), 6.6 (60 min), and 6.2 mmol/l (90 min). The corresponding insulin mean values are: 63 (basal), 200 (15 min), and 215 pmol/l (30 min).

Lipid analysis: Serum levels of triglycerides, total cholesterol, and HDL cholesterol were determined with Vitros TRIG Slides, Vitros CHOL Slides (Johnson & Johnson Clin. Diagn. Inc., USA), and Liquid N-geneous HDL-c reagent kit (Biomed-RK, Jönköping, Sweden), respectively.

Lipogenesis and Lipolysis: Male rats (75 days) were decapitated after carbon dioxide anesthesia, and epididymal fat depots (1-2 g) were removed. Adipocytes were prepared as described by Kamel et al., Pediatr. Res., 1997, 41:563-567. Studies of glucose incorporation into lipids (lipogenesis) were performed at a glucose concentration of 1 TM, at which glucose transport into the cells is rate limiting. Adipocytes were incubated at 2% (v/v) concentration in 0.5 ml Krebs Ringer phosphate buffer (KRP) containing 40 mg/ml albumin (Sigma Chemical Co., St. Louis, MO), 0.2 TM [^{3}H]-glucose (5 x 10^6 cpm), 1.0 TM unlabeled glucose, and insulin at the indicated concentrations. At each insulin concentration, the analysis was performed in triplicate at 370C for 2 h, and the reactions were terminated by rapid chilling to 40C. Incorporation of glucose into lipids was determined, as described by Moody et al., Horm. Metab. Res., 1974, 6:12-16, by mixing 45 Tl of 6.0 M H_2SO_4 and 4.0 ml of toluene with 2,5-diphenyloxazole (PPO) and adding to each vial containing adipocytes. Vials were left at room temperature overnight before liquid scintillation counting. For characterization of lipolysis, adipocytes were incubated for 2 h at 370C in KRP buffer containing 40 mg/ml albumin (Sigma) and 5.6 mmol/l glucose. The final adipocyte suspension was 1% (v/v). At the end of the incubation, an aliquot of the medium was removed for analysis of glycerol release, which was

used as an index of lipolysis. To assess maximal lipolysis, noradrenaline (1 nmol/l to 0.1 mmol/l) was added to the incubation media. Lipogenesis and lipolysis were expressed per cell surface area in order to eliminate differences solely depending on adipocyte size. Maximal insulin induced lipogenesis was calculated as the difference between glucose incorporation at maximum minus the incorporation of glucose in the absence of insulin. Maximal noradrenaline induced stimulations of lipolysis (responsiveness) were calculated from each individual dose-response curve as the maximum glycerol release minus glycerol release in the absence of noradrenaline. The concentration of noradrenaline or insulin that produced 50% of the maximum effect (EC_{50}, sensitivity) was calculated from the individual dose-response curves.

Insulin mRNA analysis: RNA levels of the rat insulin genes, *Ins1 and Ins2*, in pancreas were determined by semi-quantitative RT-PCR. Five-months old male rats were fasted for 7 h and pancreas were isolated directly or after glucose challenge. In the latter case, glucose (2 g/kg and subsequently 1 g/kg body weight) was injected intraperitoneally at 0 and 60 min, and the rats were sacrificed at 120 min. Total pancreatic RNA (0.75 Tg) was reverse transcribed in a total volume of 20 Tl, using BRL Superscript II (Life Technologies), as described by the manufacturer. The two transcripts from *Ins1* and *Ins2* were reverse transcribed with a primer common to both insulin genes (5'-TTTATTCATTGCAGAGGGGT-3', SEQ ID NO:1). The cDNA reaction (5 Tl) was directly introduced into a 25 Tl PCR solution containing Dynazyme DNA polymerase and buffer (Finnzymes Oy). *Ins1 and Ins2* genes were amplified in separate reactions with ^{32}P labeled specific primers (*Ins1* primers: 5'-GTGACCAGCTACAATCATAG-3', SEQ ID NO:2, and 5'-GTGCCAAGGTCTGAAGATCC-3', SEQ ID NO:3; *Ins2* primers: 5'-GTGACCAGCTACAGTCGGAA-3', SEQ ID NO:4, and 5'-GTGCCAAGGTCTGAAGGTCA-3', SEQ ID NO:5) by denaturation at 940C for 3 min, followed by 20 cycles consisting of denaturation at 940C for 30 s, annealing at 620C for 30 s, and extension at 720C for 1 min, with a final extension for 7 min at 720C. Insulin specific products accumulated exponentially up to cycle 24. Samples (15 Tl) were separated on 6% polyacrylamide gels, which were dried and the radioactivity visualized and quantified by phosphorimager analysis (Fujix BAS 1000).

Genotype analysis and localization of markers: Rats were genotyped by PCR amplification of microsatellite markers as previously described by Jacob, H.J. et al., Cell, 1991,

67:213-224, with the exception that ^{33}P-KATP was used to label one primer in each pair. For the genetic mapping of new markers, 45 rats with the most extreme glucose values from the first F2 intercross of GK and F344 rats were genotyped, and markers were placed on a genetic map using the computer package Mapmaker/exp 3.0.

Generation of new RFLP markers and Southern blot analysis: Hybridization probes were synthesized by RT-PCR or genomic PCR, using available rat cDNA sequences and gene specific primers. Total RNA was prepared as previously described. Six Tg of RNA was transcribed using BRL Superscript II (Life Technologies), as described by the manufacturer. For the *Jak2* probe, total RNA prepared from the whole body of a 1 day old rat was used in the reverse transcriptase reaction (cDNA primer: 5'-AAGGGCCCGTGGACACGAG-3', SEQ ID NO:6) and 2 Tl of the reverse transcriptase reaction was introduced in the subsequent PCR amplification (primers: 5'-AAGGGCCCGTGGACACGAG-3', SEQ ID NO:6, and 5'-GAAGAGCAAAAGCCCACCTG-3', SEQ ID NO:7), using a PCR-profile of denaturation at 96°C for 4 min, followed by 35 cycles consisting of denaturation at 96°C for 30 s, annealing at 55°C for 1 min, extension at 72°C for 2 min, and a final extension for 7 min at 72°C. The *jak2* gene was mapped by a HindIII RFLP with fragment lengths of 8.6 kb in GK and 6.4 kb in F344. *Pnlip* mRNA from total pancreatic RNA was reverse transcribed using a primer having the nucleotide sequence of 5'-ACTACAGAAGTTGAACACTCTG-3' (SEQ ID NO:8). PCR conditions were identical to the *jak2* reaction, except that an annealing temperature of 50°C was used (primers: 5'-CGATGCCCAGTTTGTGGATG-3', SEQ ID NO:9, and 5'-ACTACAGAAGTTGAACACTCTG-3', SEQ ID NO:10). One Tl from the first amplification was used as template in a second nested PCR (primers: 5'-ACTTAGGATTTGGAATGAGC-3', SEQ ID NO:11 and 5'-TTGGGTAGAGTTGGGTTGAT-3', SEQ ID NO:12; conditions as for *Jak2*, except that annealing was performed at 530C). A StuI RFLP was used to genetically map the *Pnlip* gene with fragments of 18 kb in GK and 14 and 4 kb in F344. The *Htr7* gene was amplified by genomic PCR at the same conditions as for *Pnlip* (primers for first PCR amplification: 5'-CGAAATCATTGGCTGAGACTG-3', SEQ ID NO:13 and 5'-GGGTACTCTTCTGAACTGTGG-3', SEQ ID NO:14; second nested PCR primers: 5'-TGGCTTCTGTCTTCTTCTTGG-3', SEQ ID NO:15 and 5'-CTGCTTCCTTACCTGTCCTTA-3', SEQ ID NO:16). An MspI RFLP was identified for *Pnlip* that generated fragments of 5.5 kb for GK and 4.5 kb for F344. Southern blot analysis was performed with high molecular weight

DNA that was extracted from rat liver and digested (10 Tg) with the appropriate restriction endonuclease. After fractionation in 0.8% agarose gels and transfer to a nylon membrane (Zeta-probe, Bio-Rad), ^{32}PK-labeled RFLP probes (random priming) were used to probe the membrane.

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Genetic mapping of Ide: The Ide probe for hybridization was synthesized by RT-PCR, using available rat cDNA sequences (GenBank Accession No. X67269 S53969) and gene specific primers. For the reverse transcriptase reaction, total RNA was prepared from the whole body of a 1 day old rat, as described above. Six Tg of RNA was transcribed in a total volume of 20 Tl using BRL Superscript II (Life Technologies), as described by the manufacturer. IDE mRNA was reverse transcribed with a primer having the nucleotide sequence 5'-AGCTGGTGGACAAACAGGAG-3', (SEQ ID NO:17) and 2 Tl of the reverse transcriptase reaction was introduced in the subsequent PCR amplification (primers: 5'-GTGAACCTGCTGATTAACTAAG-3', SEQ ID NO:18, and 5'-AGCTGGTGGACAAACAGGAG-3', SEQ ID NO:17). The PCR-profile that was used included denaturation at 940C for 4 min and 30 cycles consisting of 94°C for 30 sec, annealing at 55°C for 1 min, and extension at 72°C for 2 min, with a final extension for 7 min at 72°C. Southern blot analysis was performed as described above. A HincII RFLP was identified that generated fragments of 2.7 kb in GK and 0.7 kb in F344.

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Sequencing of rat IDE cDNA: A 3128 bp rat *Ide* cDNA fragment that was amplified by RT-PCR with gene specific primers, was sequenced. Six Tg of total RNA prepared from rat liver was used in a 20 Tl reverse transcriptase reaction with a cDNA primer having the nucleotide sequence of 5'-CTGTTTGTCTCTCTAATTGC-3' (SEQ ID NO:19). Two Tl of the reverse transcriptase reaction was introduced in the PCR reaction, using Expand Long Template PCR System (Boehringer Mannheim) as described by the manufacturer (PCR primers: 5'-ATGCGGAACGGGCTCGTGTG-3', SEQ ID NO:20, and 5'-AGCCAGAAACTACTCAAAGC-3', SEQ ID NO:21, using a PCR profile of 94°C for 2 min, and 30 cycles consisting of 94°C for 10 sec, 54°C for 30 sec, 68°C for 2.5 min, of which the last 20 cycles were elongated for 20 sec at 68°C for each cycle, and a final extension for 7 min at 68°C). The DNA sequences of the RT-PCR products were determined using ABI PRISM BigDye Terminator Cycle Sequencing Ready

Reaction kit and Ide specific primers in an ABI PRISM 377 semi-automatic sequencer (Applied Biosystems, USA).

Plasmid construction and COS1 cell transfections: Ide mRNA from GK and F344 was amplified by RT-PCR, as described above, with primers extended with restriction sites. The resulting 3.1 kb cDNA product containing the complete translated region, was ligated into BglII and MluI restriction sites of expression vector pCMV4 (D.W Russel, Dept. of Mol. Gen., University of Texas Southwestern Medical center), under control of the cytomegalovirus promoter. The Ide cDNA inserts in the resulting constructs pCMV4-Ide(GK) and pCMV4-Ide(F344) were sequenced to exclude PCR artifacts. Internal restriction sites were used to separate the GK sequence variants generating pCMV4-Ide(H18R) and pCMV4-Ide(A890V). Approximately $6x10^6$ COS-1 cells were transiently transfected by electroporation (Bio-Rad Gene Pulser, Richmond, CA; 1200 V, 25 TF) with 10 Tg of pCMV4-Ide plasmid together with 1 Tg of the 9-galactosidase vector pCH110 (Pharmacia, Sweden).

Assay of insulin degrading activity: Transfected COS-1 cells were seeded in 6 cm petri dishes, and incubated for 36 h in Dulbecco's modified Eagles medium (DMEM) supplemented with 10% calf serum. Subsequently, the cells were washed twice in PBS, preincubated at 37°C in 3 ml DMEM supplemented with 1 mg/ml BSA for 30 min, and incubated in 2 ml DMEM containing 1 mg/ml BSA and 15,000 cpm/ml of ^{125}I-insulin (ratio of labeled to unlabeled insulin was 1:150). Triplicate aliquots of 100 Tl were removed at 30, 45, and 60 min, after addition of insulin, and undegraded insulin was precipitated for 30 min on ice with one volume of 25% TCA. Samples were centrifuged at 14,000 rpm for 20 min, the supernatant recovered, and the amount of undegraded insulin measured by radioactive counting. Cells were further washed two times with PBS, incubated for 2 hours in DMEM, trypsinized, and washed 3 times in PBS. The cells (approximately $3x10^6$ cells per plate) were recovered for homogenization by sonication for 15 sec in 300 Tl of 100 mM phosphate buffer (pH=7.4) containing 0.5 mg/ml BSA. The homogenate was centrifuged at 350 g for 10 min, and the supernatant was collected for measurement of insulin degradation activity, protein concentration (Bradley ANDREJ), 9-galactosidase activity (Maniatis ANDREJ), and Western blot analysis. Triple aliquots of cell lysates containing 1 Tg protein were incubated for 15 min at 370C in 100 Tl of assay buffer

containing 2,000 cpm of ^{125}I insulin, and the amount of degraded insulin were measured as above. In all experiments background COS1 insulin degrading activity (in cells transfected with pCMV plasmid) was 20 to 25% of cells expressing wild type rat IDE. The IDE protein was detected by immunoblotting according to standard procedures using IDE antibodies kindly provided by Dr. M.R. Rosner (ANDREJ Adress).

Example 2 - Characterization of Niddm1 sub-loci: A breeding protocol was established to allow for the transfer of the GK-*Niddm1* diabetes susceptibility allele onto the background genome of the normoglycemic F344 rat. A long interval was transferred from GK to F344 to assure that no susceptibility genes in this chromosomal region were lost (Fig. 1). The GK-specific region in the congenic strain F344.GK-*Niddm1a* (*Niddm1a*) was 52∀3 cM long and contained the complete 20 cM 95% confidence interval previously defined for *Niddm1* flanked by approximately 15 cM of additional GK alleles. A number of sub-strains were produced from *Niddm1a* to define the location of the *Niddm1* susceptibility gene/genes. Two of these strains, F344.GK-*Niddm1b* (*Niddm1b*) and F344.GK-*Niddm1i* (*Niddm1i*); retained 28∀1 cM and 22∀1 cM of the GK interval. The GK regions in *Niddm1b* and *Niddm1i* are distinct and non-overlapping since two markers (Cyp2c12 and D1Mgh29), separating the two GK regions, are homozygous for F344 alleles (Fig. 1). All congenic strains were passed through 10 successive generations of backcrossing to obtain genetically pure animals. To verify the purity of the strains, a genome-wide analysis was performed with 111 markers spaced at an average of 20 cM. Special care was taken to analyze known loci for diabetes-associated phenotypes. No remaining GK derived alleles were found.

The IPGTT was used to identify the *Niddm1* locus in the original F2-intercross and also applied to characterize the congenic strains. To challenge further the animals, the IPGTT was performed in older rats (95 days compared with 70 days). *Niddm1a* rats with the complete *Niddm1* chromosome region (52 cM) differed significantly from F344 rats in glucose tolerance during IPGTT (Fig. 2A). As compared with F344, the glucose AUC were significantly higher in *Niddm1a* ($p = 0.0007$), *Niddm1b* ($p = 0.002$), and *Niddm1i* ($p = 0.00001$). The serum insulin levels at 15 and 30 min were significantly lower in *Niddm1i* than in F344 ($p = 0.01$ and 0.002). No differences in body weight were observed in this experiment when comparing *Niddm1a*, *Niddm1b*, or *Niddm1i* with F344 rats. The most pronounced difference was observed 15 min

after glucose injection, when the mean glucose concentration in *Niddm1a* was 4.0 mmol/l (26%) higher than in F344 (p = 0.0005). Also, the two congenic strains carrying separate parts of the *Niddm1* locus, displayed significantly higher postprandial glucose concentrations compared with control F344 rats.

The results of the IPGTT of *Niddm1b* and *Niddm1i* compared with the control F344 rat are shown in Figure 2B. At 15 min following glucose injection, *Niddm1b* and *Niddm1i* rats exhibited 2.3 mmol/l (15%) and 4.7 mmol/l (31%) higher glucose levels than F344 (p = 0.008 and p = 0.00005). The sum of the AUC increases over F344 for the two sub-strains (*Niddm1b* and *Niddm1i*) were distinctly larger than the AUC increase of the parental strain (*Niddm1a*). The sum AUCs of *Niddm1b* and *Niddm1i* was 325, compared to 171 in *Niddm1a*, clearly indicating that non-allelic interaction (epistasis) is operating within the *Niddm1* locus.

A discriminating feature of *Niddm1i* in comparison with *Niddm1b* was the significantly lower serum insulin levels at 15 and 30 min (p = 0.03 and p = 0.002). At 15 and 30 min post-injection, the insulin values in *Niddm1i* were 385 pmol/l (27%, p = 0.012) and 294 pmol/l (24%, p = 0.002) lower than in F344 (Fig. 2D). No significant differences in insulin levels were observed when comparing either *Niddm1a* or *Niddm1b* with F344 (Fig. 2C and D). It appears that the *Niddm1* locus contains at least two separate genes affecting glucose homeostasis, since the *Niddm1i* and *Niddm1b* both affect glucose levels but cover different parts of chromosome 1 and display major differences in glucose stimulated insulin secretion in vivo.

Example 3 - Diabetes development in *Niddm1b* and *Niddm1i*: To further investigate the diabetes phenotype associated with the GK alleles at the *Niddm1b* and *Niddm1i* loci, congenic rats were studied at different ages in a prospective study. In order to characterize the phenotypic effects of each GK-allele at the loci, GK/F344 heterozygous animals also were studied. The heterozygous animals were produced by backcrossing of *Niddm1b* or *Niddm1i* to F344. These animals were denoted *Niddm1b*/F344 and *Niddm1i*/F344, to indicate the heterozygous nature at each locus. Male rats carrying the *Niddm1b* or *Niddm1i* locus in homozygous (GK/GK) or heterozygous (GK/F344) form and F344 rats were subjected to IPGTT at 65 and 95 days of age. At 185 days of age, the basal levels of blood glucose, serum insulin,

triglyceride, total cholesterol, and HDL cholesterol were determined; subsequently the animals were sacrificed, and the epididymal fat depots were weighed.

At 65 days of age, *Niddm1b* and *Niddm1b*/F344 showed slightly elevated postprandial glucose levels (mmol/l) at the early time points (15 and 30 min) during the IPGTT as compared to F344 rats (Table 1). However, the basal and 30 min serum insulin levels (pmol/l) were significantly higher in *Niddm1b* and *Niddm1b*/F344 (Table 1).

TABLE 1

Diabetes-associated phenotypes in *Niddm1* congenics and F344 at age 65 days

Phenotype	F344 (n = 15)	*Niddm1b*/F344 (n=12)	*Niddm1b* (n=11)	*Niddm1i*/F344 (n=8)	*Niddm1i* (n=11)
Weight (g)	207 ∀ 3	216 ∀ 4	228 ∀ 4***	202 ∀ 4	197 ∀ 5
Glucose 0 min	4.8 ∀ 0.1	4.6 ∀ 0.1	4.8 ∀ 0.1	4.6 ∀ 0.2	4.7 ∀ 0.1
Glucose 15 min	15.9 ∀ 0.4	17.0 ∀ 0.5	17.3 ∀ 0.6*	15.4 ∀ 1.0	17.2 ∀ 0.5*
Glucose 30 min	9.4 ∀ 0.3	10.3 ∀ 0.4	10.6 ∀ 0.5*	8.2 ∀ 0.7	10.5 ∀ 0.4*
Glucose 60 min	4.9 ∀ 0.2	5.2 ∀ 0.3	5.5 ∀ 0.2	6.0 ∀ 0.1**	4.9 ∀ 0.1
Glucose 90 min	4.8 ∀ 0.2	5.2 ∀ 0.2	5.1 ∀ 0.1	5.6 ∀ 0.2*	4.8 ∀ 0.2
Glucose AUC	705 ∀ 12	755 ∀ 18*	774 ∀ 20**	712 ∀ 26	748 ∀ 18
Insulin 0 min	77 ∀ 7	109 ∀ 9**	122 ∀ 22*	78 ∀ 10	62 ∀ 18
Insulin 15 min	1,234 ∀ 182	1,259 ∀ 178	1,263 ∀ 143	1,111 ∀ 163	542 ∀ 76**
Insulin 30 min	498 ∀ 68	857 ∀ 64	980 ∀ 153**	351 ∀ 74	398 ∀ 68

All values are given as mean ± standard error of the mean (sem).

Each congenic strain was compared with F344 (Student=s T-test) and significant differences are indicated: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Basal insulin levels (pmol/l) in *Niddm1b* and *Niddm1b*/F344 were 58% and 42% higher compared with F344 and at 30 min post-injection, the corresponding increases were 97% and 72%. In concordance with the experiment shown in Figure 2, postprandial glucose levels (mmol/l) in mid-aged (95 days) *Niddm1b* rats were significantly higher than in F344 (Table 2).

TABLE 2

Diabetes-associated phenotypes in *Niddm1* congenics and F344 at age 95 days

Phenotype	F344 (n = 15)	*Niddm1b*/F344 (n = 12)	*Niddm1b* (n = 11)	*Niddm1i*/F344 (n = 8)	*Niddm1i* (n = 11)
Weight (g)	279 ∀ 4	280 ∀ 4	305 ∀ 5***	275 ∀ 4	270 ∀ 6
Glucose 0 min	5.1 ∀ 0.1	4.9 ∀ 0.1	5.6 ∀ 0.3	5.3 ∀ 0.1	5.8 ∀ 0.2**
Glucose 15 min	15.1 ∀ 0.5	15.4 ∀ 0.6	17.4 ∀ 0.7**	16.6 ∀ 1.1	18.5 ∀ 0.8***
Glucose 30 min	12.2 ∀ 0.3	12.2 ∀ 0.5	14.1 ∀ 0.7**	13.1 ∀ 0.4	14.0 ∀ 0.4***
Glucose 60 min	7.5 ∀ 0.3	7.2 ∀ 0.3	8.1 ∀ 0.5	7.3 ∀ 0.3	8.5 ∀ 0.3*
Glucose 90 min	6.1 ∀ 0.2	6.0 ∀ 0.2	7.1 ∀ 0.2**	6.6 ∀ 0.2	7.2 ∀ 0.2***
Glucose AUC	855 ∀ 21	846 ∀ 29	971 ∀ 35**	901 ∀ 31	1001 ∀ 28***
Insulin 0 min	210 ∀ 27	208 ∀ 23	238 ∀ 80	260 ∀ 38	225 ∀ 48
Insulin 15 min	1,425 ∀ 205	1,589 ∀ 141	1,166 ∀ 287	1,787 ∀ 142	810 ∀ 193*
Insulin 30 min	1,200 ∀ 186	1,507 ∀ 138	1,141 ∀ 297	1,563 ∀ 168	792 ∀ 189

All values are given as mean " standard error of the mean (sem).

Each congenic strain was compared with F344 (Student's T-test) and significant differences are indicated: $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

No difference in glucose levels was observed between *Niddm1b*/F344 and F344. At this age, the serum insulin levels in the heterozygous animals were still slightly higher (15 and 30 min). In contrast, a slight insulin decrease was observed in the homozygous animals (Table 2). Although, the insulin levels in *Niddm1b* were not significantly different from F344 during IPGTT, insulin secretion was impaired in light of the increased glucose levels.

Later in life (185 days), both basal glucose and basal insulin levels in *Niddm1b* were significantly higher than in F344 rats (Table 3). The levels of triglyceride and HDL cholesterol also were significantly higher in *Niddm1b* than in F344 rats (Table 3), while the total cholesterol levels were not different. In contrast to the cholesterol levels in *Niddm1b* rats, both total cholesterol and HDL cholesterol levels in the heterozygous rats (*Niddm1b*/F344) were significantly lower than in F344 rats. No differences in basal glucose, insulin, or triglyceride levels were found between *Niddm1b*/F344 and F344. Moreover, the *Niddm1b* rats were significantly heavier (10%, 9%, and 6%, at 65, 95, and 185 days, respectively) than F344 rats in this experimental series (Table 1-3), and the epididymal fat weight was increased by 18% (Table 3). The increase in *Niddm1b* body weight was not observed in the first experiment, the possibility that this reflects merely a consequence of differences in nutrition during early life can not be excluded. Genetic linkage to body weight, however, was observed in the original genetic analysis of the GK rat in the region corresponding to *Niddm1b*. See, Galli et al., 1996, *supra*.

TABLE 3

Diabetes-associated phenotypes in *Niddm1* congenics and F344 at age 185 days

Phenotype	F344 (n = 9)	*Niddm1b*/F344 (n = 11)	*Niddm1b* (n = 10)	*Niddm1i*/F344 (n = 8)	*Niddm1i* (n = 10)
Weight (g)	365 ∀ 9	354 ∀ 4	389 ∀ 4*	356 ∀ 4	350 ∀ 6
Basal glucose (mmol/l)	5.7 ∀ 0.1	5.6 ∀ 0.2	6.2 ∀ 0.1**	5.9 ∀ 0.1	5.7 ∀ 0.1
Basal insulin (pmol/l)	378 ∀ 71	423 ∀ 48	631 ∀ 38**	408 ∀ 47	472 ∀ 50
Fat weight (g)	10.5 ∀ 0.7	9.4 ∀ 0.2	12.4 ∀ 0.3*	9.8 ∀ 0.4	10.2 ∀ 0.4
Triglyceride (mmol/l)	2.30 ∀ 0.09	2.07 ∀ 0.13	3.03 ∀ 0.16**	2.25 ∀ 0.09	2.16 ∀ 0.14
Total cholesterol (mmol/l)	2.12 ∀ 0.05	1.87 ∀ 0.03***	2.22 ∀ 0.05	2.18 ∀ 0.02	2.31 ∀ 0.08
HDL cholesterol (mmol/l)	0.96 ∀ 0.02	0.87 ∀ 0.02**	1.05 ∀ 0.03*	1.05 ∀ 0.02**	1.08 ∀ 0.04**

All values are given as mean ∀ standard error of the mean (sem).

Each congenic strain was compared with F344 (Student's T-test) and significant differences are indicated: *P<0.05, **P<0.01, ***P<0.001.

In *Niddm1i* rats, the postprandial glucose levels at 95 days were significantly higher when compared with F344 rats (Table 2). Furthermore, similar to the first experimental series (Fig. 2), serum insulin levels during IPGTT were lower in *Niddm1i* rats (Table 2). Also, in 65 day old *Niddm1i* rats, the insulin levels were lower compared with F344 rats, indicating a pronounced and early B-cell defect in *Niddm1i*. At 15 min post glucose-injection, insulin levels in *Niddm1i* rats were 56% of that in F344 rats, despite slightly elevated blood glucose levels (Table 1). No major differences in glucose or insulin levels were found between *Niddm1i*/F344

and F344 rats at 65 or 95 days. At the age of 185 days, neither *Niddm1i* nor *Niddm1i*/F344 differed from F344 for any of the analyzed phenotypes, except for higher HDL cholesterol in both *Niddm1i* and *Niddm1i*/F344 (Table 3).

Example 4 - Insulin action in adipocytes: To characterize further the *Niddm1* phenotype, adipocytes were isolated from the epididymal fat depot of rats at age 75 days (*Niddm1i*, *Niddm1b*, F344, and GK). Lipogenesis was determined as incorporation of radioactive glucose into lipids in response to increasing concentrations of insulin. Compared with F344 rats, adipocytes from both *Niddm1b* and *Niddm1i* rats had significantly lower basal and insulin induced lipogenesis, but were significantly higher than adipocytes from GK rats, which demonstrated severely reduced insulin action (Fig. 3). There was no significant difference between the congenic strains *Niddm1b* and *Niddm1i*. The EC_{50} of insulin induced lipogenesis revealed no inter-strain difference in insulin sensitivity. In addition, lipolysis was studied by measuring glycerol release from isolated adipocytes. No significant differences were observed in either basal lipolysis or noradrenaline induced lipolysis. This demonstrates that the observed differences in insulin action reflect a pathway-specific defect and not a general adipocyte dysfunction.

Example 5 - Candidate gene function and syntenic conservation: The insulin 1 gene (*Ins1*) is located in the GK interval contained in *Niddm1i* and was a candidate for mutations causing the impaired glucose homeostasis. A difference in the *Ins1* promoter sequence between GK and F344 rats has been reported at nucleotide position -258 bp relative to the transcription start site, although both strains contain a similar relative abundance of *Ins1* and *Ins2* mRNA in pancreas. Galli et al., 1996, *supra*. To investigate a potential role of this genetic variation in more detail, the pancreatic levels of steady state mRNA for *Ins1* and *Ins2* were estimated by semiquantitative RT-PCR in GK, F344, and *Niddm1i* rats (n=4) after a fasting period of 7 h and after 2 h of repeated glucose injections (Fig. 4). Compared with F344, the total insulin mRNA level was 30% higher in *Niddm1i* rats, in spite of the impaired insulin response demonstrated during IPGTT. The relative expression of *Ins1* and *Ins2*, however, did not differ among the strains in either the basal or the glucose stimulated state. Thus, *Ins1* is excluded as a candidate for the *Niddm1i* phenotype. The insulin RNA data show that the defect in insulin secretion observed in *Niddm1i* is located downstream of the regulation of insulin transcription.

Information of the corresponding homologous regions to *Niddm1* in human and mouse is important for locating candidate genes, and for comparisons of the *Niddm1* rat locus with other susceptibility loci linked to type II diabetes or its associated phenotypes in the human or the mouse. Roughly guided by previously mapped genes on rat chromosome 1 and conserved synteni between rat, human, and mouse, three new genes were mapped to the *Niddm1* locus on rat chromosome 1. These were the genes encoding Janus kinase 2 (JAK2), 5-hydroxytryptamine receptor 7 (HTR7), and pancreatic lipase (PNLIP) (indicated in bold in Fig. 1). This demonstrates homology between the *Niddm1* locus and human chromosome region 9p24 and, furthermore, confirms the syntenic conservation between rat chromosome 1, human chromosome region 10q24-26, and mouse chromosome 19 (Table 4).

TABLE 4

Genes in the Niddm1 region on rat chromosome 1 and localization of the human and mouse homologues*

Gene Name	Gene Symbol	Chromosomal Localization		
		Rat[1]	Human	Mouse[2]
Glutathione-S-transferase, pi	Gstp	118	11q13	19 (0)
Phosphorylase, glycogen; muscle	Pygm	118	11q13.1	19 (2)
Janus kinase 2	Jak2	133	9p24	19 (24)
5-hydroxytryptamine receptor 7	Htr7	140	10q24	19 (33)
Cytochrome P450, subfamily Iic	Cyp2c	142	10q24.1	19 (27)
Glutamic-oxaloacetic transaminase 1	Got1	144	10q24.1-25.1	19 (37)
Insulin 1	Ins1	154	NA	19 (49)
Pancreatic lipase	Pnlip	159	10q26.1	19 (29)

* Background information was obtained from Rat Genome Database (http://ratmap.gen.gu.se/), Mouse Genome Informatics (http://www.informatics.jax.org/), and Genome Database (http://www.gdb.org/).

1 Distance in cM from marker D1Mgh2, located in the centromeric end of rat chromosome 1.

2 Distance in cM from the centromere is shown in parentheses preceded by the chromosome number

Example 6 - Congenic substrains and associated phenotypes: For further characterization of *Niddm1b*, congenic sub-lines were established. *Niddm1b* rats were backcrossed to F344 and recombinants were identified within the GK interval. Three recombinants, covering distinct parts of the GK interval were selected, and homozygous lines were established for the GK-allele. The resulting congenic strains, F344.GK-*Niddm1c* (*Niddm1c*), F344.GK-*Niddm1f* (*Niddm1f*), and F344.GK-*Niddm1e* (*Niddm1e*) maintained 23∀1 cM, 7.6∀1 cM, and 3.7∀2 cM, respectively, of GK alleles (Fig. 5).

Since an intraperitoneal glucose tolerance test was used to identify *Niddm1* as well as to define the *Niddm1b* and *Niddm1i* sub-loci, the same test was applied to characterize the *1c*, *1e*, and *1f* strains. To map the susceptibility gene within *Niddm1b*, rats from the new congenic sub-strains (*Niddm1e*, *Niddm1f*, and *Niddm1c*) and F344 were subjected to IPGTT at 95 days of age. Similarly to *Niddm1b*, postprandial glucose levels in both *Niddm1e* and *Niddm1f* were significantly higher than in F344 (Table 5). The most pronounced difference was observed at 30 min after glucose injection, when the glucose levels were 21% higher in both *Niddm1e* and *Niddm1f*. The basal and 30 min insulin levels also were significantly higher than in F344. No significant differences in glucose or insulin levels were observed between *Niddm1c* and F344.

TABLE 5

***Niddm1e, Niddm1f, Niddm1c* and F344**

Phenotype	F344 (n=12)	*Niddm1e* (n=10)		*Niddm1f* (n=11)		*Niddm1c* (n=11)	
	Mean ∀ sem	Mean ∀ sem	P value	Mean ∀ sem	P value	Mean ∀ sem	P value
65 days rats							
Weight (g)	223 ∀ 4	195 ∀ 2	0.00001	211 ∀ 4	NS	201 ∀ 7	0.009
Glucose 0 min	5.6 ∀ 0.2	5.6 ∀ 0.2	NS	5.9 ∀ 0.2	NS	5.5 ∀ 0.1	NS
Glucose 15 min	16.6 ∀ 0.7	15.7 ∀ 0.9	NS	17.5 ∀ 0.7	NS	17.3 ∀ 0.3	NS
Glucose 30 min	10.1 ∀ 0.5	10.2 ∀ 0.7	NS	10.6 ∀ 0.4	NS	9.0 ∀ 0.4	NS
Glucose 60 min	5.4 ∀ 0.1	5.2 ∀ 0.2	NS	5.7 ∀ 0.2	NS	5.3 ∀ 0.1	NS
Glucose 90 min	5.4 ∀ 0.1	5.4 ∀ 0.5	NS	6.1 ∀ 0.2	0.008	5.7 ∀ 0.2	NS
Insulin 0 min	114 ∀ 13	102 ∀ 16	NS	145 ∀ 10	NS	75 ∀ 12	0.04
Insulin 15 min	2039 ∀ 194	1335 ∀ 195	0.02	2039 ∀ 146	NS	1436 ∀ 289	NS
Insulin 30 min	1112 ∀ 321	725 ∀ 137	NS	1159 ∀ 166	NS	749 ∀ 163	NS
95 days rats							
Weight (g)	268 ∀ 4	276 ∀ 3	NS	277 ∀ 5	NS	258 ∀ 5	NS
Glucose 0 min	5.3 ∀ 0.1	5.4 ∀ 0.1	NS	5.7 ∀ 0.1	0.01	5.6 ∀ 0.2	NS
Glucose 15 min	15.4 ∀ 0.4	17.2 ∀ 0.7	0.03	17.3 ∀ 0.7	0.03	15.6 ∀ 0.6	NS
Glucose 30 min	12.0 ∀ 0.3	14.5 ∀ 0.3	0.00005	14.5 ∀ 0.5	0.0002	11.8 ∀ 0.5	NS
Glucose 60 min	6.8 ∀ 0.1	8.4 ∀ 0.3	0.0001	7.7 ∀ 0.2	0.0008	6.1 ∀ 0.2	0.01
Glucose 90 min	6.3 ∀ 0.1	6.7 ∀ 0.1	NS	6.8 ∀ 0.3	NS	6.6 ∀ 0.2	NS
Insulin 0 min	201 ∀ 25	331 ∀ 24	0.001	441 ∀ 37	0.0003	246 ∀ 57	NS
Insulin 15 min	2162 ∀ 167	2425 ∀ 102	NS	2431 ∀ 215	NS	2265 ∀ 373	NS
Insulin 30 min	1626 ∀ 164	2497 ∀ 93	0.0003	2405 ∀ 162	0.004	1554 ∀ 320	NS
120 days rats							
Weight (g)	303 ∀ 4	314 ∀ 4	NS	309 ∀ 5	NS	NA	
Basal glucose	5.8 ∀ 0.1	5.0 ∀ 0.1	0.0001	5.3 ∀ 0.2	0.02	NA	
Basal insulin	276 ∀ 23	386 ∀ 55	NS	390 ∀ 36	0.01	NA	

For further characterization, the congenic strains were studied after treatment with a diet containing a high amount of fat. *Niddm1e*, *Niddm1f*, and F344 rats were treated with the high fat diet described in Example 1, starting at the age of 120 days. Rats were subjected to IPGTT and the basal levels of triglyceride, total cholesterol, and HDL cholesterol were determined at 225 days of age; subsequently the animals were sacrificed, and the epididymal fat depots were weighed. At this age, postprandial glucose levels were still significantly higher in *Niddm1e* and *Niddm1f* as compared with F344 (Table 6). In contrast to the IPGTT at 95 days, the differences were more pronounced at the later time points after glucose injection (Table 5). At 90 min after injection, the glucose levels in both congenics were approximately 30% higher than in F344. The basal insulin levels were significantly higher in the congenics than in F344, but the insulin levels after glucose injection were not. At this age, increases in both body weight and epididymal fat weight were observed, however, were only significantly increased in *Niddm1e*.

TABLE 6

Phenotype	F344 (n=12)	*Niddm1e*(n=10)		*Niddm1f(n=11)*	
	Mean ∀ sem	Mean ∀ sem	P value	Mean ∀ sem	P value
Rats 185 days					
Weight	373 ∀ 5	386 ∀ 4	NS	379 ∀ 7	NS
Basal glucose	4.5 ∀ 0.2	5.3 ∀ 0.1	0.001	5.2 ∀ 0.8	0.02
Basal insulin	459 ∀ 42	553 ∀ 71	NS	578 ∀ 24	0.03
Rats 220 days					
Weight (g)	377 ∀ 6	400 ∀ 5	0.01	384 ∀ 8	NS
Glucose 0 min	4.8 ∀ 0.1	4.9 ∀ 0.1	NS	4.9 ∀ 0.1	NS
Glucose 15 min	17.1 ∀ 0.3	20.3 ∀ 1.3	0.04	17.1 ∀ 1.4	NS
Glucose 30 min	17.7 ∀ 0.8	19.4 ∀ 0.6	NS	18.5 ∀ 0.7	NS
Glucose 60 min	14.6 ∀ 0.7	17.6 ∀ 1.0	0.02	17.8 ∀ 0.6	0.005
Glucose 90 min	10.4 ∀ 0.4	13.9 ∀ 0.9	0.0009	13.4 ∀ 0.8	0.001
Insulin 0 min	337 ∀ 16	480 ∀ 46	0.003	410 ∀ 24	0.02
Insulin 15 min	1069 ∀ 109	1166 ∀ 143	NS	985 ∀ 111	NS
Insulin 30 min	1217 ∀ 77	2533 ∀ 144	NS	1049 ∀ 71	NS
230 days rats					
Weight (g)	368 ∀ 4	391 ∀ 6	0.004	375 ∀ 8	NS
Fat weight	8.5 ∀ 0.3	10.6 ∀ 0.5	0.0009	9.3 ∀ 0.4	NS
Basal insulin	314 ∀ 31	413 ∀ 24	0.02	454 ∀ 47	0.02
Cholest	4.35 ∀ 0.13	4.33 ∀ 0.20	NS	4.13 ∀ 0.15	NS
Trig	0.66 ∀ 0.03	0.76 ∀ 0.06	NS	0.61 ∀ 0.02	NS
HDL	1.23 ∀ 0.07	0.97 ∀ 0.02	0.003	1.12 ∀ 0.03	NS
LDL	2.80 ∀ 0.08	3.00 ∀ 0.17	NS	2.77 ∀ 0.16	NS

As described in Example 2, basal and insulin-induced lipogenesis in *Niddm1b* was significantly reduced as compared with F344. A similar test was performed with the *Niddm1b* sub-strains, *Niddm1e* and *Niddm1f*, and control F344. Lipogenesis also was reduced in both *Niddm1e* and *Niddm1f* compared with F344 (Fig. 6). Based on these data, the *Niddm1b* diabetes susceptibility gene/genes is located in the 3.7 cM GK interval of *Niddm1e*.

Example 7 - DNA sequence analysis and expression of Ide: Candidate genes were identified using genetic mapping data from humans and mice. Synteny is conserved between the *Niddm1* region on rat chromosome 1 and human chromosomes 9 and 10 and mouse chromosome 19. One gene that has not previously been considered a candidate for diabetes is the gene encoding insulin degrading enzyme (IDE), which mapped to human chromosome 10q24 and mouse 19. The Ide gene was genetically mapped on rat chromosome 1 within the GK interval of *Niddm1e*, by restriction fragment length polymorphism (RFLP) analysis (Figure 5).

To investigate the possibility that changes in the IDE protein structure could explain the phenotype of *Niddm1e*, the cDNA sequence of IDE was determined in both GK and F344 rats. Sequencing the complete translated part of the gene revealed three nucleotide differences between GK and F344 rats, one in the 5'-end (codon 18) and two in the 3' end (codons 890 and 934) of the coding region (Fig. 7). Two of these resulted in amino-acid changes, a CAC to CGC change at codon 18 resulted in the substitution of Arginine for histidine and a GCG to GTG transition at codon 890 resulted in the substitution of valine for alanine. The third variant was silent, changing the last base of codon 934 (GAT to GAC). Additionally, the IDE cDNA sequences were determined in 12 other rat strains (DA, PVG/RT1, PVG/Bk, Lew, ACI, BN, Cop, BB, W, SD,FRL, and FSL). The A890V variation was unique for GK, while H18R was also found in the strains, DA, ACI, SD, FRL, and FSL (Table 7).

TABLE 7

Sequence variants in the Ide gene of various rat strains

Strain	Codon 18	Codon 890	Codon 934
GK	CGC (Arg)	GTG (Val)	GAC
F344	CAC (His)	GCG (Ala)	GAT
PVG	CAC (His)	GCG (Ala)	GAT
LEW	CAC (His)	GCG (Ala)	GAT
BN	CAC (His)	GCG (Ala)	GAT
COP	CAC (His)	GCG (Ala)	GAT
BB	CAC (His)	GCG (Ala)	GAT
Wistar	CAC (His)	GCG (Ala)	GAT
DA	CGC (Arg)	GCG (Ala)	GAT
ACI	CGC (Arg)	GCG (Ala)	GAT
SD	CGC (Arg)	GCG (Ala)	GAC
FRL	CGC (Arg)	GCG (Ala)	GAC
FSL	CGC (Arg)	GCG (Ala)	GAC

To study the effect of the GK variant of IDE, insulin degrading activity was determined in an *in vitro* expression system. IDE was over-expressed in transfected COS1 cells and the ability of cell lysates to hydrolyze insulin were studied. Western blot analysis with anti-IDE antibodies confirmed expression of transfected IDE cDNAs. The two amino-acid variations in the GK allele, H18R and A890V, were studied separately or in combination. Insulin degrading activity in intact cells transfected with the GK allele containing both H18R and A890V, was decreased by 34% ($p<0.001$) compared with control (Fig. 8). When the two variants were analyzed separately, only H18R showed slightly reduced activity (89%) of wild-type, $p<0.001$ as compared with control, while A890V was normal, indicating a synergistic effect of the two variants. In cell lysates, no differences were observed for the GK allele on insulin degradation compared to the normal variant.

Niddm1e displayed elevated postprandial glucose levels, impaired basal and insulin induced lipogenesis in isolated adipocytes, increased body weight and epididymal fat mass, and hyperinsulinemia. Furthermore, *Niddm1e* were treated with high fat diet under a period of 3.5 months and subsequently the rats were subjected to IPGTT at the age of 7.5 months. At this age, the most pronounced difference in glucose levels as compared with F344 were observed at the later time points after glucose injection and not as in younger animals at the earlier time points. Thus, the diabetes locus *Niddm1b* is redefined to a 3 cM region in the congenic strain *Niddm1e*.

These data indicate that a gene encoding IDE partly explains the diabetic phenotype in the GK rat and through some of its multiple actions in the cell, causes hyperglycemia and insulin resistance in *Niddm1e*. Several other studies have shown decreased insulin clearance associated with insulin resistance and diabetes, suggesting that a reduction in insulin degradation could mediate a diabetic phenotype. A possible molecular explanation is that in peripheral tissues, a decreased intra-cellular degradation of insulin bound to its receptor could inhibit the re-circulation of the insulin receptor back to the cell membrane and thus lowering the number of available receptors on the cell membrane.

Example 8 – NiddmC congenic animals: Genome-wide linkage analysis, as described in Galli et al. 1996, *supra*, was used to localize chromosome regions demonstrating genetic linkage to diabetes-associated phenotypes. The F2-population of intercross progeny generated between GK and F344 rats was analyzed with all F2 animals together, and separately for sex and reciprocal cross, in favor of linkage in a dense, genome-wide search for linkage to diabetes-associated phenotypes. Table 8 describes loci with a LOD (logarithm of odds ratio) greater than 3 for at least one diabetes-associated phenotype. The marker located in the middle of each chromosome region is shown in Table 8, and is located in the center of approximately 25 cM, which encompass each of these QTLs for diabetes-associated phenotypes.

Congenic animals of the *NiddmC* series (NiddmC2, NiddmC3, NiddmC5, NiddmC7, NiddmC9A, NiddmC9B, NiddmC10, NiddmC11, NiddmC13, NiddmC18, NiddmC(13+15), and NiddmC(9+13+15)) were generated by backcrossing GK onto F344 and choosing progeny which

had lost maximum GK derived (donor) alleles in each generation (marker assisted selection, see, Whittaker et al., Genet Res., 66(3):255-65, 1995;and Darvasi, Nat Genet., 18(1):19-24, 1998.

The microsatellite markers listed in Table 9 cover QTLs identified in F2 progeny from intercrossing GK and F344. Theses markers distinguish between GK and F344 alleles and display the GK-allele after backcrossing to generate congenic animals. The diabetes-associated QTLs, which were selected for during generation of the respective congenic strain, are listed in Table 9, followed by the microsatellite markers that define GK-derived alleles within each QTL. All other tested markers outside this selected chromosome region exhibited an F344-specific genotype. These background markers were located approximately every 50 cM along the rat genome and were specifically selected against by choosing the progeny in each backcross generation that had lost the maximum amount of GK-derived (donor) background.

Table 10 provides a summary of the metabolic syndrome QTLs that regulate phenotypes associated with type II diabetes (obesity, insulin resistance, glucose intolerance, and dyslipemia). Phenotypes linked to a chromosome region are noted in the F2 population. Congenic animals have been generated for each of the noted loci, and the phenotype confirmed in at least five different congenic animals.

TABLE 8

List of Markers with a LOD score >3

Chromosome	Marker at LOD max	Highest LOD-score for diabetes-associated phenotype
C1	D1Mit9	4.0
c1	D1Mgh40	3.2
c1	D1Mit18	3.6
c1	D1Mit34	7.3
c1	D1Mgh25	8.7
c1	GTREPB	3.2
c1	D1Mit7	8.0
c1	D1Mgh24	8.5
c1	D1Mgh13	3.6
c1	D1Mit8	5.7
c1	D1Mit14	3.0
c2	D2Mit11	4.6
c2	D2Mit14	3.2
c2	D2N91	3.4
c3	D3Mit8	3.2
c3	D3Rat27	3.1
c4	D4Mit28	3.9
c7	D7Mit28	4.4
c7	D7Rat27	8.6
c7	D7Rat106	3.8
c7	D7Mit6	7.4
c7	D7Mgh23	3.1
c7	D7Mit11	3.5
c7	D7Mit9	3.1
c9	D9Mgh3	4.6
c9	D9Rat104	6.9
c10	D10Rat64	3.3
c10	D10Mit8	4.1
c10	D10Mgh23	5.2
c10	D10Mgh5	5.4
c10	D13Mit11?	3.8
c12	D12Rat22	3.6
c13	D13Mgh16	4.6
c15	D15Rat25	3.1
c17	D17Mgh6	3.7
c18	D18Mit11	4.2
c19	D19Mgh10	3.5
c20	D20Mit5	3.2
c20	D20Rat29	4.7
x	DXMgh8	4.9
x	DXRat16	5.5
x	DXRat20	7.0
x	DXRat103	4.2

TABLE 9

NiddmC Congenic Animals

NiddmC2:	D2Mgh5, D2Mgh15, D2Mit10, D2Mit11, D2Mgh30, D2Mit22, D2Mgh11, and D2Arb24.
NiddmC3:	D3Mgh19, D3Mit10, and D3Mgh8,and D3Mgh6..
NiddmC5:	D5Mgh5, D5Mit10, D5Mit2, D5Mit11, D5Mit4, D5Mit5, and D5Mgh23.
NiddmC7:	D7Mgh11, D7Mit23, D7Mit7, D7Mit22, D7Mit6, D7Mgh10, and D7Mit5.
NiddmC9A:	D9Mgh3, D9Mit4, D9Mit2, IGFBP5X, and GDNPN1.
NiddmC9B:	D9Mgh3, and D9Mit4.
NiddmC10:	D10Mit15, D10Mit16, D10Mit18, D10Mit9, D2Mit11, D10Mgh6, D10Mit13, D10Mgh5, D10Mit12, D10Mgh4, and D10Mit11.
NiddmC11:	D11Mgh5, D11Mgh4, D11Mgh3, and D11Mgh2.
NiddmC13:	D13Mgh16, D13Mgh2, D13Mit2, and D13Mit5.
NiddmC18:	D18Mit4, D18Mgh5, D18Mgh11, D18Mgh6, and D18Mit11.
NiddmC(13+15)	D13Mgh16, D13Mgh2, D13Mit2, D13Mit5, BMYO, D15Mgh15, D15Mgh8, and D15Mco2
NiddmC(9+13+15):	D9Mit4, D9Mit2, D13Mit2, D13Mit5, D15Mgh8, and D15Mgh9.

TABLE 10

Summary of QTLS and Congenic Animals

Chr	Locus name[1]	Phenotype linked to chromosome-region in F2-population	Congenic generated, size in cM	Confirmed phenotype in congenic animals	Candidate gene-variant	Functional consequence of gene-variant
1	Niddm1e	PHG, W[2]	Yes, 1.9-5.5	PHG, FI, O[2], HDL	Ide (H18R+A890V)	30% reduced activity
1	Niddm1i	PHG, PI	Yes, 18.3-18.8	PHG, PI	–	–
2	Niddm2	W, PHG	Yes, 69-109	–	–	–
3	"NiddmC3"	W, PI, PHG	Yes, 30-50	–	–	–
5	"NiddmC5"	W, PHG	Yes, 35-73	W	–	–
7	Weight1	W	Yes, 49-58	W	–	–
9	"NiddmC9"	FI, PI	Yes, 35-71	W, PHG	–	–
10	Niddm3	FHG, PHG W, C, TG	Yes, 81-111	–	–	–
11	"NiddmC11"	FHG, FI	Yes, 35-64	–	–	–
13	"NiddmC13"	PHG	Yes, 40-66	–	–	–
15	"NiddmC15"	W, PI	Yes, 8-39	–	–	–
18	"NiddmC18"	W, PHG, FI	Yes, 43-47	–	–	–

1) Citation marks indicate tentative name

2) Weight (W) is related to the more exact phenotype obesity (O) that is measured by dissecting the epididymal fat pad and weighing of the isolated tissue.

PHG, postprandial hyperglycemia

FHG, fasting hyperglycemia

FI, fasting insulin concentration or insulin to glucose ratio

PI, postprandial insulin concentration or insulin to glucose ratio

W, weight

O, increased fat weight

C, cholesterol

TG, triglyceride

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

What is claimed is:

1. A non-human congenic animal comprising genetic material of a donor animal and a recipient animal, said congenic animal exhibiting a type II diabetes-associated phenotype, wherein less than about one chromosome of said congenic animal's genome is derived from said donor animal, and wherein said genetic material from said donor is necessary for expression of said type II diabetes-associated phenotype in said congenic animal.

2. The animal of claim 1, wherein said congenic animal is marker-defined.

3. The animal of claim 1, wherein less than about 50 cM of said congenic animal's genome is derived from said donor.

4. The animal of claim 1, wherein less than about 20 cM of said congenic animal's genome is derived from said donor.

5. The animal of claim 1, wherein less than about 10 cM of said congenic animal's genome is derived from said donor.

6. The animal of claim 1, wherein less than about 5 cM of said congenic animal's genome is derived from said donor.

7. The animal of claim 1, wherein said type II diabetes-associated phenotype is selected from the group consisting of elevated postprandial glycemia, hypertension, glucose intolerance, insulin resistance, abnormal insulin secretion, reduced insulin action, increased body weight, dyslipidemia, hyperinsulinemia, impaired lipogenesis, altered glycogen metabolism, altered coagulation atherosclerosis, altered kidney function, altered nerve function, altered eye function, obesity, and inflammation.

8. The animal of claim 1, wherein said donor animal's genome comprises a *Niddm1a* genomic interval.

9. The animal of claim 1, wherein said congenic animal's genome derived from said donor comprises a *Niddm1e* genomic interval.

10. The animal of claim 1, wherein said congenic animal's genome derived from said donor is selected from a genomic interval selected from the group consisting of *Niddm1a, Niddm1b, Niddm1c, Niddm1d, Niddm1e, Niddm1f, Niddm1g, Niddm1h,* and *Niddm1i.*

11. The animal of claim 1, wherein said congenic animal's genome derived from said donor is selected from a genomic interval selected from the group consisting of *NiddmC2, NiddmC3, NiddmC5, NiddmC7, NiddmC9A, NiddmC9B, NiddmC10, NiddmC11, NiddmC13, NiddmC18, NiddmC(13+15)*, and *NiddmC(9+13+15)*.

12. The animal of claim 1, wherein substantially all mitochondria of said congenic animal are derived from either said recipient animal or said donor animal.

13. The animal of claim 1, wherein substantially all mitochondria of said congenic animal are derived from said recipient.

14. An isolated cell of the congenic animal of claim 1.

15. The cell of claim 14, wherein said cell is selected from the group consisting of adipocytes, mesangial cells, hepatic cells, pancreatic cells, muscle cells, endothelial cells, and neural cells.

16. A tissue culture derived from the congenic animal of claim 1.

17. The tissue culture of claim 16, wherein said culture is selected from the group consisting of adipose tissue, mesangial tissue, hepatic tissue, pancreatic tissue, muscle tissue, blood-vessel tissue, and neural tissue.

18. The congenic animal of claim 1, wherein said congenic animal is selected from the group consisting of non-human mammals, insects, and birds.

19. The congenic animal of claim 1, wherein said non-human mammal is a rodent or a swine.

20. The congenic animal of claim 19, wherein said rodent is a rat, mouse, or guinea pig.

21. The congenic animal of claim 20, wherein said rodent is a rat.

22. A non-human congenic animal population comprising a plurality of non-human congenic animals, said congenic animals exhibiting a plurality of type II diabetes-associated phenotypes, wherein each congenic animal within said plurality of congenic animals comprises genetic material from a donor animal and a recipient animal, wherein about 0.1% to about 50% of each congenic animal's genome is derived from said donor animal, and wherein said genetic

material from said donor is necessary for expression of said type II diabetes-associated phenotype in each said congenic animal.

23. A method for testing a pharmaceutically active compound comprising the steps of:

a) administering a test compound to a non-human congenic animal exhibiting a type II diabetes-associated phenotype, wherein said non-human congenic animal comprises genetic material of a donor animal and a recipient animal, wherein less than about 50 cM of said congenic animal's genome is derived from said donor animal, and wherein said genetic material from said donor is necessary for expression of said type II diabetes-associated phenotype in said congenic animal; and

b) evaluating said test compound for an effect on at least one type II diabetes-associated phenotype in said congenic animal.

24. The method of claim 23, wherein said congenic animal comprises a *Niddm1a* genetic interval.

25. The method of claim 23, wherein said congenic animal comprises a *Niddm1e* genetic interval.

26. The method of claim 23, wherein said congenic animal comprises a *Niddm1* genetic interval selected from the group consisting of *Niddm1a*, *Niddm1b*, *Niddm1c*, *Niddm1d*, *Niddm1e*, *Niddm1f*, *Niddm1g*, *Niddm1h*, and *Niddm1i*.

27. The method of claim 23, wherein said animal comprises a progeny animal of a cross between two congenic parent animals, said parent animals having distinct congenic intervals.

28. A method for testing a pharmaceutically active compound comprising the steps of:

a) administering a test compound to a plurality of non-human congenic animals exhibiting a plurality of type II diabetes-associated phenotypes; and

b) evaluating said test compound for an effect on at least one type II diabetes-associated phenotype, wherein each congenic animal within said plurality of congenic animals comprises genetic material from a donor animal and a recipient animal, wherein about 0.1% to

about 50% of each congenic animal's genome is derived from said donor animal, and wherein said genetic material from said donor is necessary for expression of said type II diabetes-associated phenotype in each said congenic animal.

29. The method of claim 28, wherein said plurality of congenic animals comprises at least two rats having congenic intervals on different chromosomes.

30. An article of manufacture comprising isolated cells of a non-human congenic animal exhibiting a type II diabetes-associated phenotype.

31. The article of manufacture of claim 30, said article further comprising a label or package insert indicating said cells are useful for evaluating compounds that may be effective for alleviating type II diabetes-associated phenotypes.

32. A method of doing business comprising the steps of:

a) offering for sale a non-human congenic animal exhibiting a type II diabetes-associated phenotype, or a cell derived therefrom; and

b) communicating that said animal is effective for testing or evaluating compounds that are effective for alleviating type II diabetes-associated phenotypes.

33. A method of making a non-human congenic animal comprising:

a) mating a donor animal and a recipient animal to produce a progeny animal; and

b) successively backcrossing said progeny animal with said recipient animal for at least 10 generations to produce said congenic animal, said congenic animal exhibiting a type II diabetes-associated phenotype, wherein less than about 50 cM of said congenic animal's genome is derived from said donor animal, and wherein said genetic material of said donor is necessary for expression of said type II diabetes-associated phenotype in said congenic animal.

34. An isolated insulin degradation polypeptide, wherein said polypeptide comprises at least one amino acid substitution, wherein said amino acid substitution is linked to a type II diabetes-associated phenotype.

35. The polypeptide of claim 34, wherein said polypeptide comprises at least one amino acid substitution in the amino acid sequence of SEQ ID NO:23.

36. The polypeptide of claim 35, wherein an arginine residue is substituted at amino acid 18 of SEQ ID NO:23.

37. The polypeptide of claim 35, wherein a valine residue is substituted at amino acid 890 of SEQ ID NO:23.

38. The polynucleotide of claim 35, wherein said polypeptide has a substitution at amino acid 18 and amino acid 890 of SEQ ID NO:23.

39. An isolated polynucleotide encoding an insulin degradation polypeptide, said polypeptide comprising an amino acid substitution, said amino acid substitution linked to a type II diabetes-associated phenotype.

40. The polynucleotide of claim 39, wherein an arginine residue is substituted at residue 18 of SEQ ID NO:23.

41. The polynucleotide of claim 39, wherein a valine residue is substituted at residue 890 of SEQ ID NO:23.

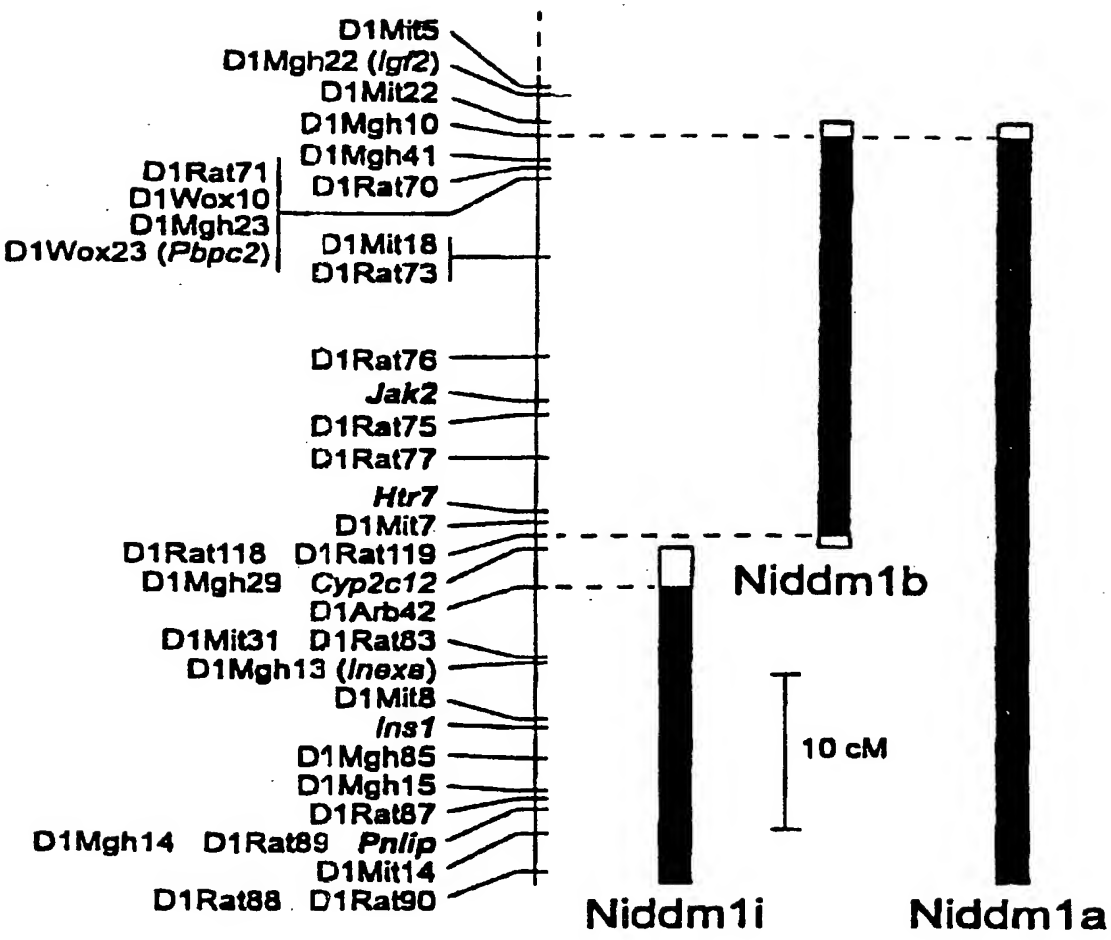
42. The polynucleotide of claim 39, wherein said polynucleotide has a cytosine residue at nucleotide 2817 of SEQ ID NO:22

43. A transgenic non-human animal whose genome comprises an insulin-degrading polypeptide transgene, said transgene comprising a regulatory polynucleotide operably linked to a polynucleotide encoding an insulin-degrading polypeptide, said insulin-degrading polypeptide having an amino acid substitution linked to a type II diabetes-associated phenotype.

44. The animal of claim 43, wherein said animal is a rat.

45. The animal of claim 43, wherein said animal is a mouse.

FIGURE 1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D1Rat70
D1Mit18
D1Rat73
D1Rat76
Jak2
D1Rat75
D1Rat77
Htr7
D1Mit7
D1Rat118
D1Rat119
D1Mgh29
Cyp2c12
D1Arb42
D1Mit31
D1Rat83
D1Mgh13 (Inexa)
D1Mit8
Ins1
D1Mgh85
D1Mgh15
D1Rat87
D1Mgh14
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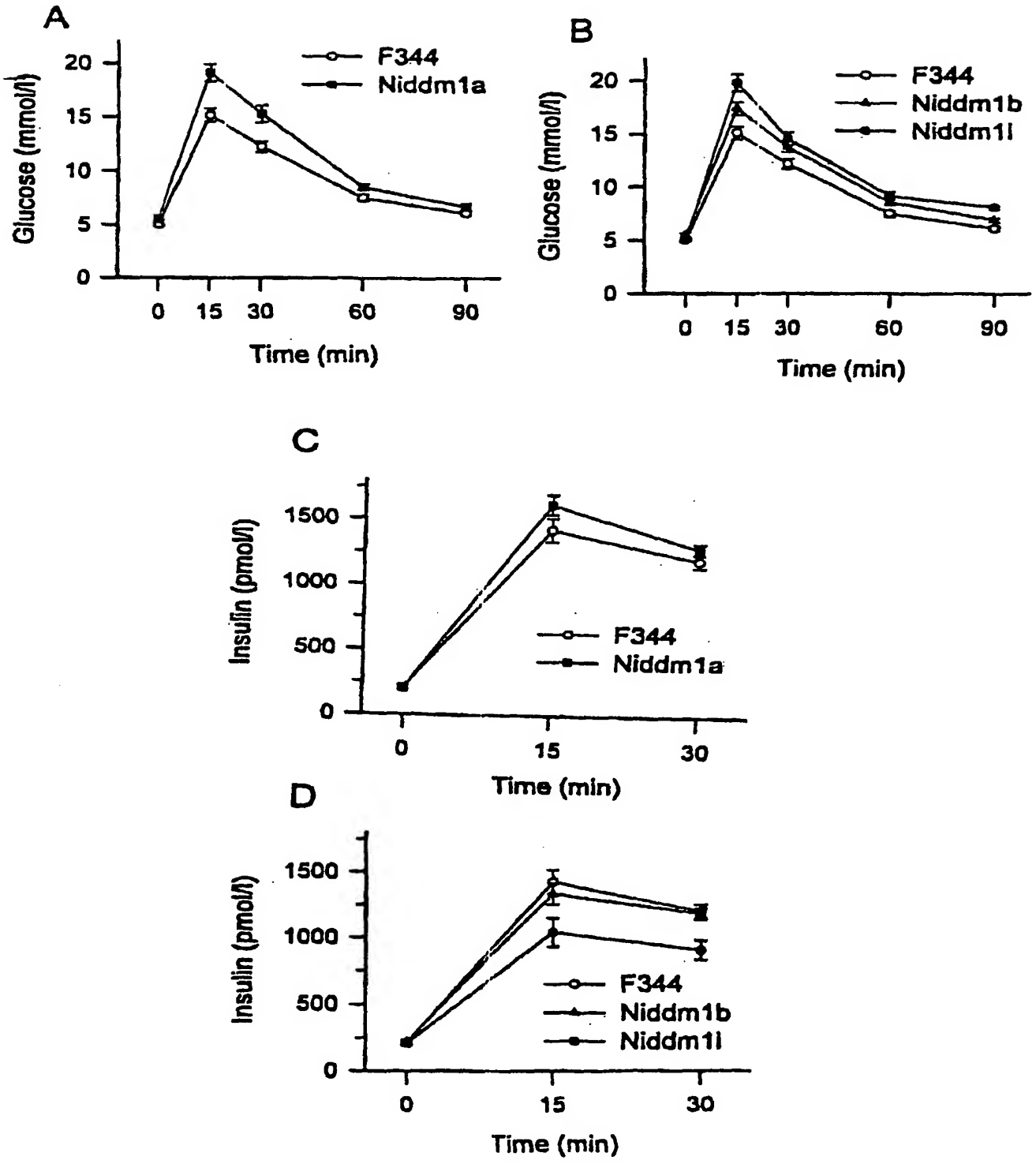
Niddm1i
Niddm1a

FIGURE 2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20
15
10
5
0
0 15 30 60 90
Time (min)
F344
Niddm1a
B
Glucose (mmol/l)
20
15
10
5
0
0 15 30 60 90
Time (min)
F344
Niddm1b
Niddm1l
C
Insulin (pmol/l)
1500
1000
500
0
0 15 30
Time (min)
F344
Niddm1a
D
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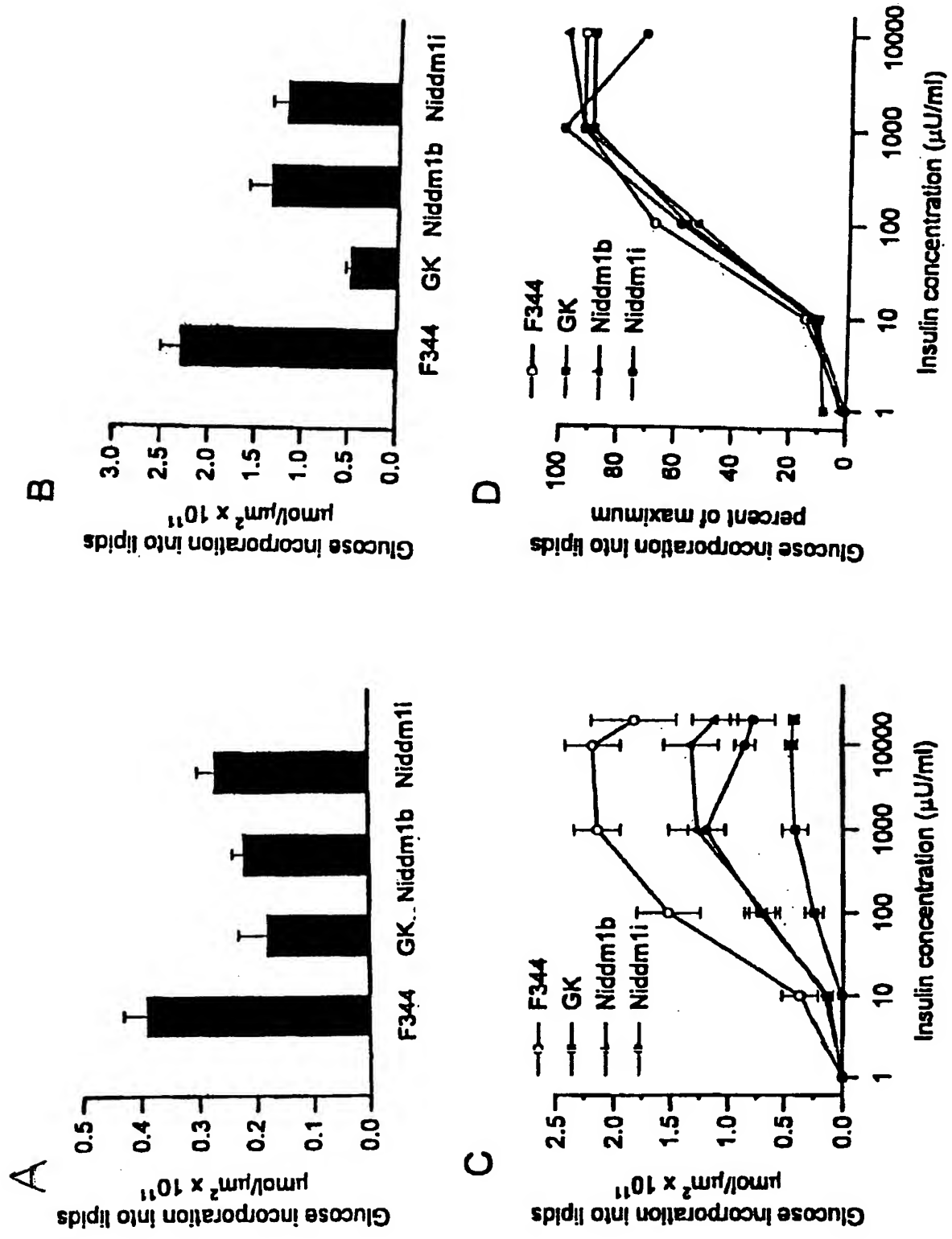
1500
1000
500
0
0 15 30
Time (min)
F344
Niddm1b
Niddm1l

FIGURE 3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GK
Niddm1b
Niddm1i
B
Glucose incorporation into lipids
μmol/μm² x 10¹¹
0.0
0.5
1.0
1.5
2.0
2.5
3.0
F344
GK
Niddm1b
Niddm1i
C
Glucose incorporation into lipids
μmol/μm² x 10¹¹
0.0
0.5
1.0
1.5
2.0
2.5
F344
GK
Niddm1b
Niddm1i
1
10
100
1000
10000
Insulin concentration (μU/ml)
D
Glucose incorporation into lipids
percent of maximum
0
20
40
60
80
100
F344
GK
Niddm1b
Niddm1i
1
10
100
1000
10000
Insulin concentration (μU/ml)

FIGURE 4

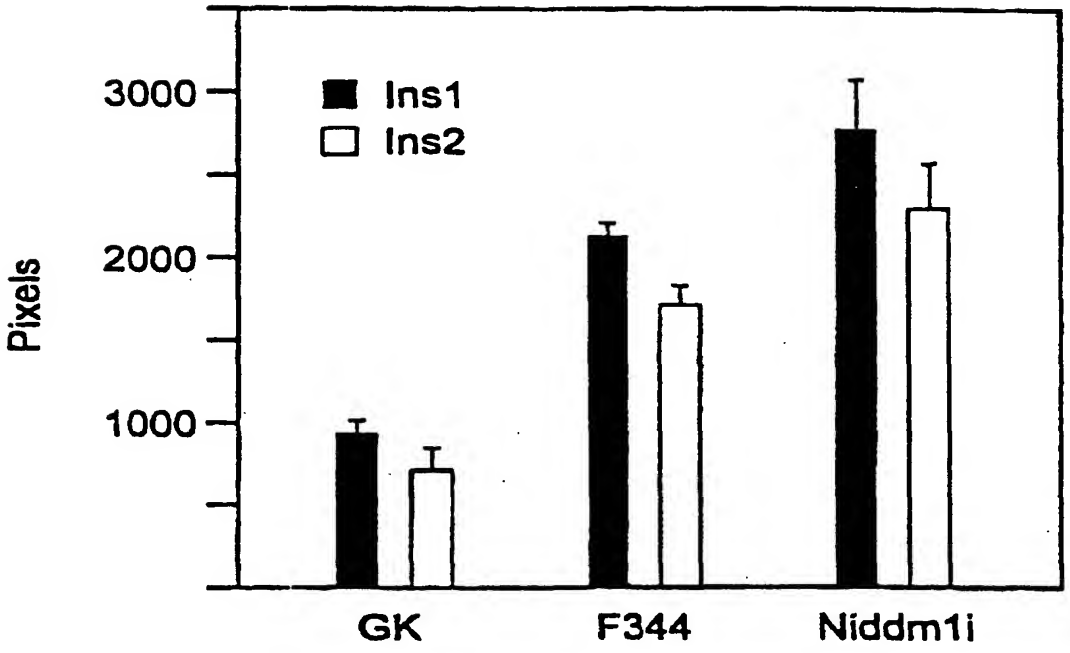
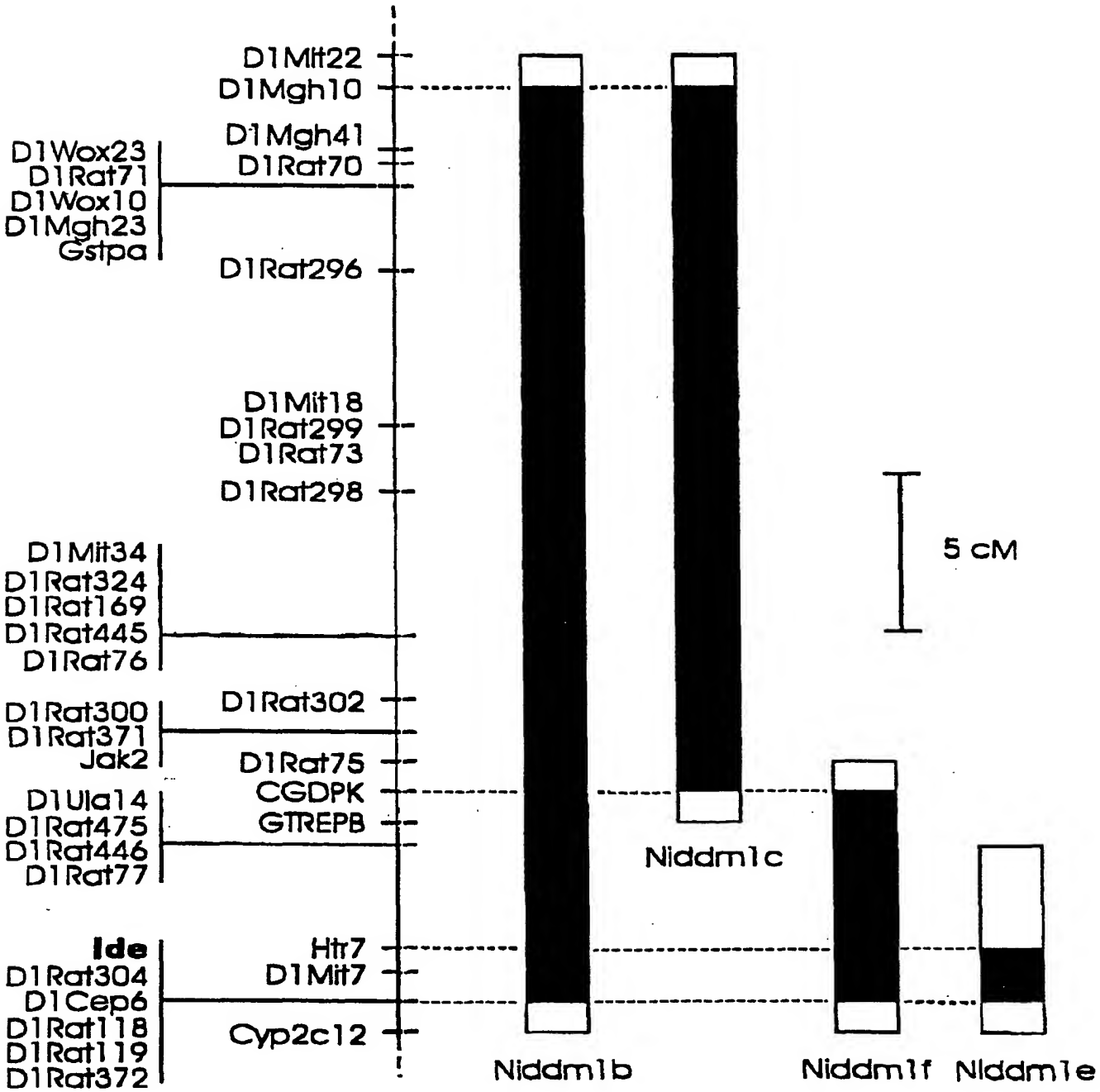


FIGURE 5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D1Rat299
D1Rat73
D1Rat298
D1Mit34
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D1Rat169
D1Rat445
D1Rat76
D1Rat302
D1Rat300
D1Rat371
Jak2
D1Rat75
CGDPK
GTREPB
D1Ula14
D1Rat475
D1Rat446
D1Rat77
Htr7
D1Mit7
Ide
D1Rat304
D1Cep6
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5 cM
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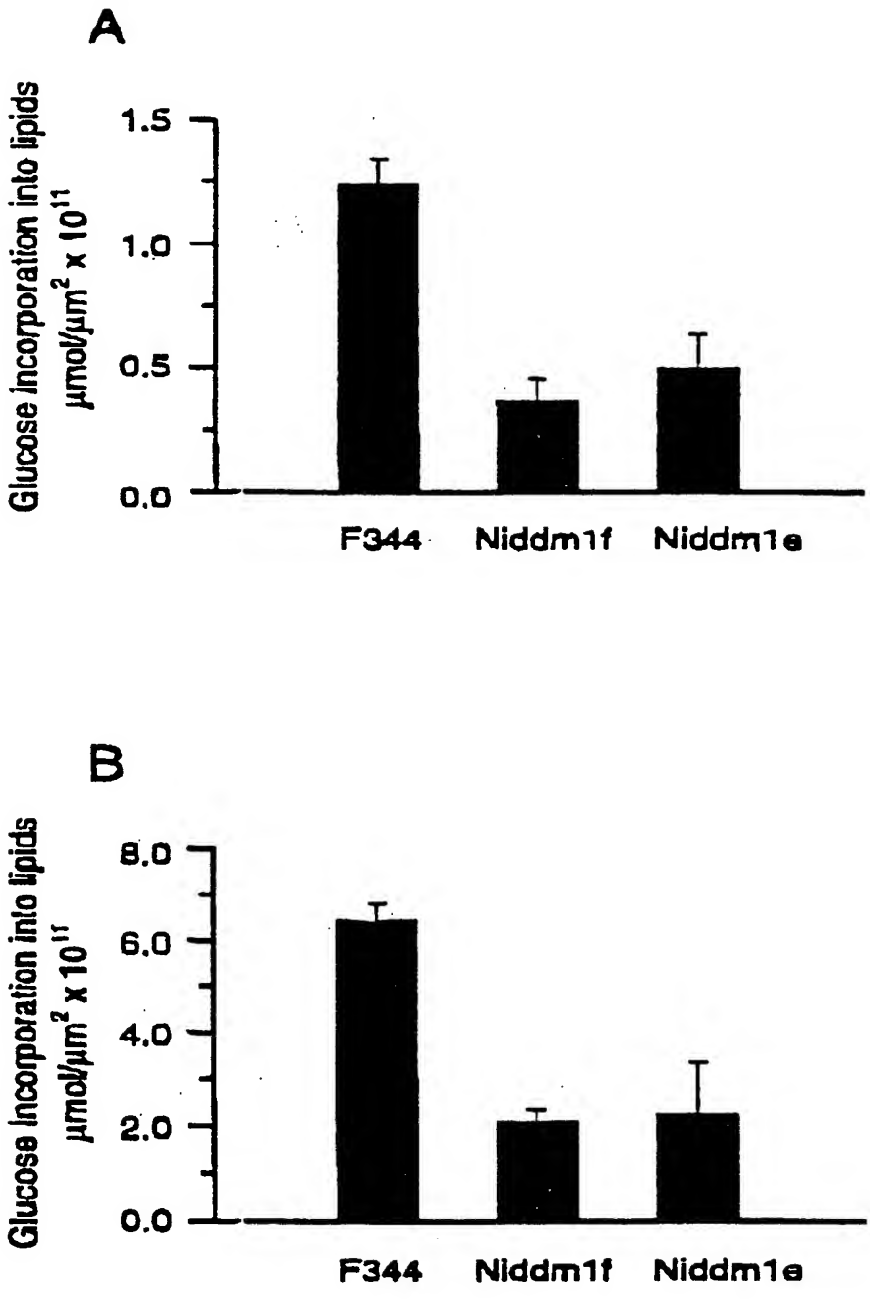
Niddm1f
Niddm1e

FIGURE 6


A
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1.0
0.5
0.0
F344
Niddm1f
Niddm1e
B
Glucose incorporation into lipids
µmol/µm² x 10¹¹
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6.0
4.0
2.0
0.0
F344
Niddm1f
Niddm1e

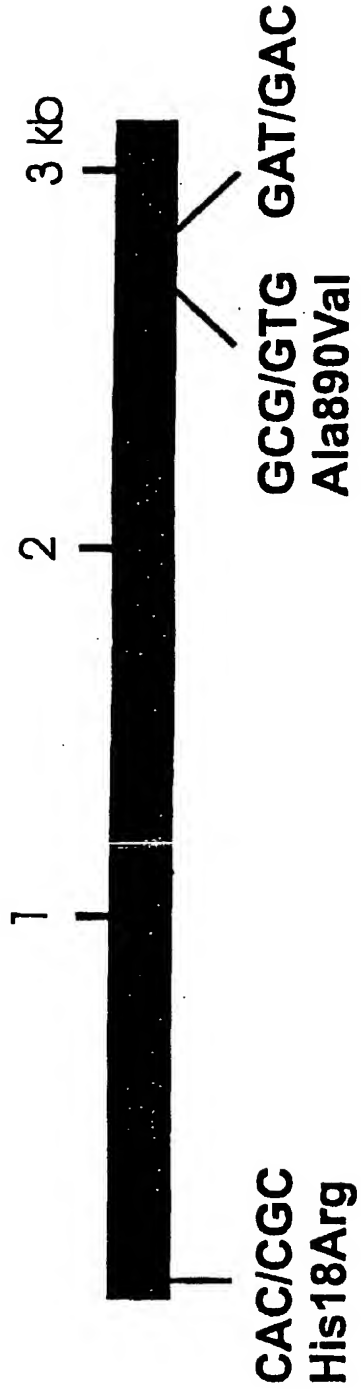
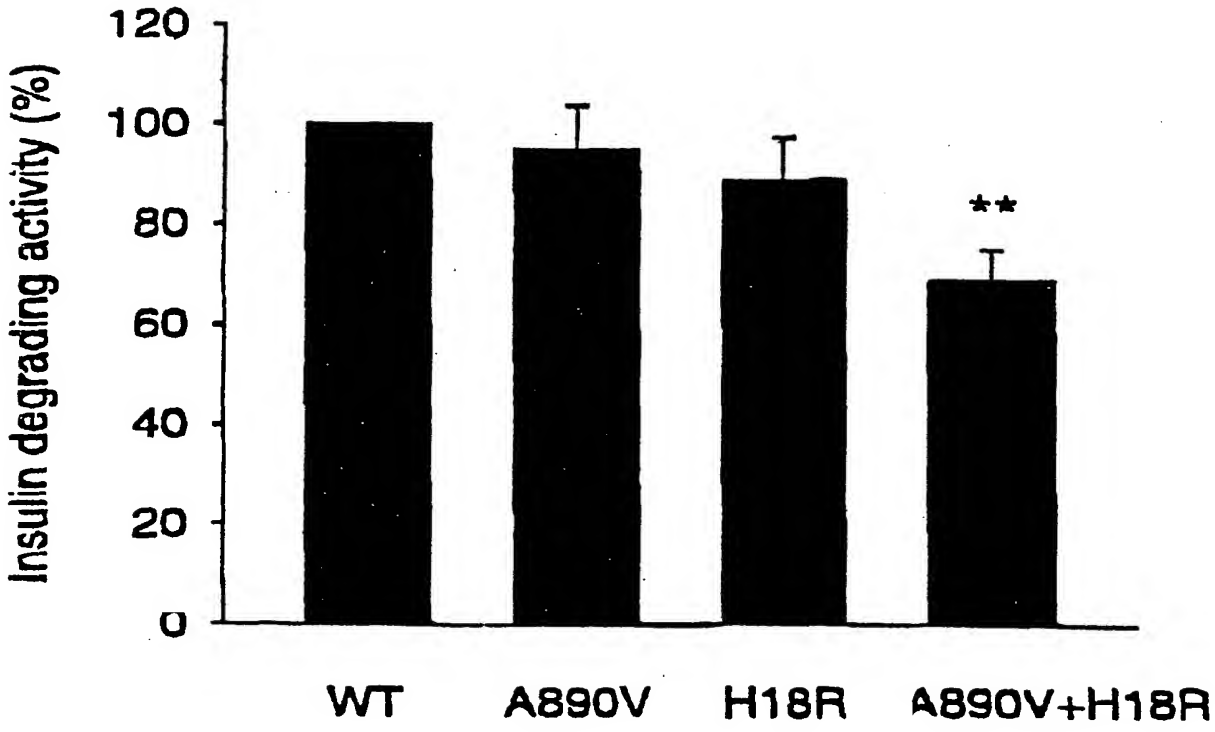


FIGURE 7

FIGURE 8


Insulin degrading activity (%)
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100
80
60
40
20
0
**
WT
A890V
H18R
A890V+H18R

SEQUENCE LISTING

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Leu Pro Ser Thr Leu His Ser Ile Leu Gly Ala Arg Pro Pro Pro Val
15 20 25

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30 35 40

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Pro Ala Ile Gln Arg Ile Glu Asp His Ile Val Lys Ser Pro Glu Asp
45 50 55 60

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Lys Arg Glu Tyr Arg Gly Leu Glu Leu Ala Asn Gly Ile Lys Val Leu
65 70 75

```
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             80                  85                  90

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His Ile Gly Ser Leu Ser Asp Pro Pro Asn Ile Pro Gly Leu Ser His
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Phe Cys Glu His Met Leu Phe Leu Gly Thr Lys Lys Tyr Pro Lys Glu
    110                 115                 120

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125                 130                 135                 140

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Phe Thr Ser Gly Glu His Thr Asn Tyr Tyr Phe Asp Val Ser His Glu
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His Leu Glu Gly Ala Leu Asp Arg Phe Ala Gln Phe Phe Leu Cys Pro
            160                 165                 170

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Leu Phe Asp Ala Ser Cys Lys Asp Arg Glu Val Asn Ala Val Asp Ser
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Glu His Glu Lys Asn Val Met Asn Asp Ala Trp Arg Leu Phe Gln Leu
    190                 195                 200

gaa aag gct aca ggg aat ccc aag cac ccc ttc agc aaa ttc ggg aca      675
Glu Lys Ala Thr Gly Asn Pro Lys His Pro Phe Ser Lys Phe Gly Thr
205                 210                 215                 220

gga aac aaa tat act cta gag act cga ccc aac caa gaa ggc atc gat      723
Gly Asn Lys Tyr Thr Leu Glu Thr Arg Pro Asn Gln Glu Gly Ile Asp
                225                 230                 235

gta agg gaa gaa ctc ttg aaa ttt cac tct acg tat tat tcg tcc aat      771
Val Arg Glu Glu Leu Leu Lys Phe His Ser Thr Tyr Tyr Ser Ser Asn
            240                 245                 250

ctg atg gcg att tgt gtt tta ggt cga gaa tct tta gat gac ctg act      819
Leu Met Ala Ile Cys Val Leu Gly Arg Glu Ser Leu Asp Asp Leu Thr
        255                 260                 265

aat ctg gtg gtg aag tta ttt tct gaa gta gag aat aaa aat gtc cct      867
Asn Leu Val Val Lys Leu Phe Ser Glu Val Glu Asn Lys Asn Val Pro
    270                 275                 280

ttg cct gaa ttt cct gag cac cct ttc caa gaa gaa cat ctt aaa caa      915
Leu Pro Glu Phe Pro Glu His Pro Phe Gln Glu Glu His Leu Lys Gln
285                 290                 295                 300
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ctt tat aaa ata gta ccc att aag gat att agg aat ctt tat gtg aca      963
Leu Tyr Lys Ile Val Pro Ile Lys Asp Ile Arg Asn Leu Tyr Val Thr
                305                 310                 315

ttt ccc ata cca gac ctt caa cag tac tac aaa tcc aat ccc ggt cat     1011
Phe Pro Ile Pro Asp Leu Gln Gln Tyr Tyr Lys Ser Asn Pro Gly His
            320                 325                 330

tat ctc ggt cat ctg atc ggg cat gaa ggt cct gga agc ctg ttg tca     1059
Tyr Leu Gly His Leu Ile Gly His Glu Gly Pro Gly Ser Leu Leu Ser
        335                 340                 345

gag ctc aag tca aag ggc tgg gta aac acc ctg gtt ggg gga cag aag     1107
Glu Leu Lys Ser Lys Gly Trp Val Asn Thr Leu Val Gly Gly Gln Lys
    350                 355                 360

gaa gga gcc cga ggt ttt atg ttt ttt atc att aat gtg gac tta act     1155
Glu Gly Ala Arg Gly Phe Met Phe Phe Ile Ile Asn Val Asp Leu Thr
365                 370                 375                 380

gaa gaa ggg tta tta cat gtt gaa gat ata att ttg cac atg ttt caa     1203
Glu Glu Gly Leu Leu His Val Glu Asp Ile Ile Leu His Met Phe Gln
                385                 390                 395

tac att cag aag cta cgt gct gaa gga cct caa gaa tgg gtt ttc caa     1251
Tyr Ile Gln Lys Leu Arg Ala Glu Gly Pro Gln Glu Trp Val Phe Gln
            400                 405                 410

gag tgc aag gac ttg aac gct gtc gct ttc agg ttt aaa gat aaa gag     1299
Glu Cys Lys Asp Leu Asn Ala Val Ala Phe Arg Phe Lys Asp Lys Glu
        415                 420                 425

agg cca cga ggc tac aca tct aag att gca ggg aaa ttg cac tat tat     1347
Arg Pro Arg Gly Tyr Thr Ser Lys Ile Ala Gly Lys Leu His Tyr Tyr
    430                 435                 440

ccc cta aat gga gtg ctc aca gct gaa tat tta ctg gaa gaa ttt aga     1395
Pro Leu Asn Gly Val Leu Thr Ala Glu Tyr Leu Leu Glu Glu Phe Arg
445                 450                 455                 460

ccc gac ttg ata gac atg gtt ctt gat aaa ctc aga cca gaa aat gtc     1443
Pro Asp Leu Ile Asp Met Val Leu Asp Lys Leu Arg Pro Glu Asn Val
                465                 470                 475

cgg gtg gca ata gtt tct aaa tca ttt gaa ggg aaa act gac cgc aca     1491
Arg Val Ala Ile Val Ser Lys Ser Phe Glu Gly Lys Thr Asp Arg Thr
            480                 485                 490

gag cag tgg tat gga acc cag tac aag caa gaa gct atc cca gag gac     1539
Glu Gln Trp Tyr Gly Thr Gln Tyr Lys Gln Glu Ala Ile Pro Glu Asp
        495                 500                 505

gtc att cag aaa tgg caa aat gct gac ctg aat ggg aaa ttt aaa ctt     1587
Val Ile Gln Lys Trp Gln Asn Ala Asp Leu Asn Gly Lys Phe Lys Leu
    510                 515                 520
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cca aca aag aat gaa ttc att cct aca aat ttt gag att ttg gca tta     1635
Pro Thr Lys Asn Glu Phe Ile Pro Thr Asn Phe Glu Ile Leu Ala Leu
525                 530                 535                 540

gaa aaa gat gca aca cca tac cct gct ctt att aag gat aca gcc atg     1683
Glu Lys Asp Ala Thr Pro Tyr Pro Ala Leu Ile Lys Asp Thr Ala Met
                545                 550                 555


agt aag ctg tgg ttc aaa caa gat gat aaa ttt ttc ttg ccg aaa gct     1731
Ser Lys Leu Trp Phe Lys Gln Asp Asp Lys Phe Phe Leu Pro Lys Ala
            560                 565                 570

tgt ctc aac ttt gaa ttt ttc agc ccg ttt gct tat gtg gac ccc ttg     1779
Cys Leu Asn Phe Glu Phe Phe Ser Pro Phe Ala Tyr Val Asp Pro Leu
        575                 580                 585

cac tgt aac atg gcc tat ttg tac ctt gaa ctc ctc aaa gac tca ctc     1827
His Cys Asn Met Ala Tyr Leu Tyr Leu Glu Leu Leu Lys Asp Ser Leu
    590                 595                 600

aac gag tat gca tat gca gca gag cta gca ggc ctg agc tat gat ctc     1875
Asn Glu Tyr Ala Tyr Ala Ala Glu Leu Ala Gly Leu Ser Tyr Asp Leu
605                 610                 615                 620

caa aac acc atc tat ggg atg tat ctc tca gtg aaa ggt tac aat gac     1923
Gln Asn Thr Ile Tyr Gly Met Tyr Leu Ser Val Lys Gly Tyr Asn Asp
                625                 630                 635

aaa cag cca att ttg cta aag aag atc acc gag aaa atg gct act ttt     1971
Lys Gln Pro Ile Leu Leu Lys Lys Ile Thr Glu Lys Met Ala Thr Phe
            640                 645                 650

gag att gat aaa aaa aga ttt gaa att atc aaa gag gcg tac atg cga     2019
Glu Ile Asp Lys Lys Arg Phe Glu Ile Ile Lys Glu Ala Tyr Met Arg
        655                 660                 665

tct ctt aat aat ttc cgg gct gag cag cct cac cag cac gcc atg tac     2067
Ser Leu Asn Asn Phe Arg Ala Glu Gln Pro His Gln His Ala Met Tyr
    670                 675                 680

tac ctc cgt ctg ctg atg act gaa gtg gcc tgg acc aaa gat gag tta     2115
Tyr Leu Arg Leu Leu Met Thr Glu Val Ala Trp Thr Lys Asp Glu Leu
685                 690                 695                 700

aaa gaa gcc ctc gat gat gtg acc ctc ccc cgt ctt aag gcc ttc ata     2163
Lys Glu Ala Leu Asp Asp Val Thr Leu Pro Arg Leu Lys Ala Phe Ile
                705                 710                 715

cct cag ctg ctg tcc cgg ctg cat att gaa gcc ctt ctc cat ggc aac     2211
Pro Gln Leu Leu Ser Arg Leu His Ile Glu Ala Leu Leu His Gly Asn
            720                 725                 730

ata aca aag cag gct gcc tta gga gtt atg cag atg gta gaa gac acc     2259
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Ile Thr Lys Gln Ala Ala Leu Gly Val Met Gln Met Val Glu Asp Thr
        735                 740                 745

ctt att gag cat gct cac acc aaa cct ctc ctt cca agt cag cta gtc     2307
Leu Ile Glu His Ala His Thr Lys Pro Leu Leu Pro Ser Gln Leu Val
    750                 755                 760

cgg tac aga gaa gtt cag ctc ccc gac cga gga tgg ttt gtt tac cag     2355
Arg Tyr Arg Glu Val Gln Leu Pro Asp Arg Gly Trp Phe Val Tyr Gln
765                 770                 775                 780

cgg agg aac gaa gtc cac aat aac tgt ggc att gag att tac tac cag     2403
Arg Arg Asn Glu Val His Asn Asn Cys Gly Ile Glu Ile Tyr Tyr Gln
                785                 790                 795

aca gac atg cag agc acc tcg gag aac atg ttc ctg gag ctc ttc tgc     2451
Thr Asp Met Gln Ser Thr Ser Glu Asn Met Phe Leu Glu Leu Phe Cys
            800                 805                 810

cag att atc tct gag cct tgc ttc aac act ctg cgt acc aag gag cag     2499
Gln Ile Ile Ser Glu Pro Cys Phe Asn Thr Leu Arg Thr Lys Glu Gln
        815                 820                 825

ctt ggc tat att gtc ttc agt gga cct cgt cgg gcc aac ggc atc cag     2547
Leu Gly Tyr Ile Val Phe Ser Gly Pro Arg Arg Ala Asn Gly Ile Gln
    830                 835                 840

ggc ttg cga ttc atc atc cag tca gaa aaa cca cct cac tac ctg gaa     2595
Gly Leu Arg Phe Ile Ile Gln Ser Glu Lys Pro Pro His Tyr Leu Glu
845                 850                 855                 860

agc aga gtg gaa gcc ttc ttg atc acc atg gaa aag gcc ata gag gac     2643
Ser Arg Val Glu Ala Phe Leu Ile Thr Met Glu Lys Ala Ile Glu Asp
                865                 870                 875

atg aca gag gag gct ttc caa aaa cac att cag gcg tta gcg att cgc     2691
Met Thr Glu Glu Ala Phe Gln Lys His Ile Gln Ala Leu Ala Ile Arg
            880                 885                 890

cga ctc gac aaa cca aag aaa ctc tct gca gag tgc gcg aag tac tgg     2739
Arg Leu Asp Lys Pro Lys Lys Leu Ser Ala Glu Cys Ala Lys Tyr Trp
        895                 900                 905

ggg gag atc atc tcc cag cag tac aat tat gac aga gat aac ata gag     2787
Gly Glu Ile Ile Ser Gln Gln Tyr Asn Tyr Asp Arg Asp Asn Ile Glu
    910                 915                 920

gtt gca tat tta aag aca ctc agc aag gat gat atc atc aaa ttc tac     2835
Val Ala Tyr Leu Lys Thr Leu Ser Lys Asp Asp Ile Ile Lys Phe Tyr
925                 930                 935                 940

aag gaa atg ttg gct gtg gac gca cca agg aga cat aaa gta tcc gtc     2883
Lys Glu Met Leu Ala Val Asp Ala Pro Arg Arg His Lys Val Ser Val
                945                 950                 955

cac gtt ctt gcc agg gaa atg gat tct tgt cct gtg gtt gga gag ttc     2931
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His Val Leu Ala Arg Glu Met Asp Ser Cys Pro Val Val Gly Glu Phe
            960                 965                 970

ccc tct cag aat gat ata aac ctt tcc gaa gcg cca ccc ttg cca caa     2979
Pro Ser Gln Asn Asp Ile Asn Leu Ser Glu Ala Pro Pro Leu Pro Gln
        975                 980                 985

cct gag gtg att cat aac atg act gaa ttc aag cgc ggc ctg ccg ctg     3027
Pro Glu Val Ile His Asn Met Thr Glu Phe Lys Arg Gly Leu Pro Leu
     990                 995                1000

ttc ccc ctt gtg aag cca cac att aac ttc atg gcg gca aaa ctc tga     3075
Phe Pro Leu Val Lys Pro His Ile Asn Phe Met Ala Ala Lys Leu  *
1005                1010                1015

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Leu His Ser Ile Leu Gly Ala Arg Pro Pro Pro Val Lys Arg Leu Cys
             20                  25                  30
Gly Phe Pro Lys Gln Ile Tyr Ser Thr Met Asn Asn Pro Ala Ile Gln
         35                  40                  45
Arg Ile Glu Asp His Ile Val Lys Ser Pro Glu Asp Lys Arg Glu Tyr
     50                  55                  60
Arg Gly Leu Glu Leu Ala Asn Gly Ile Lys Val Leu Leu Ile Ser Asp
 65                  70                  75                  80
Pro Thr Thr Asp Lys Ser Ser Ala Ala Leu Asp Val His Ile Gly Ser
                 85                  90                  95
Leu Ser Asp Pro Pro Asn Ile Pro Gly Leu Ser His Phe Cys Glu His
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                100                 105                 110
Met Leu Phe Leu Gly Thr Lys Lys Tyr Pro Lys Glu Asn Glu Tyr Ser
        115                 120                 125
Gln Phe Leu Ser Glu His Ala Gly Ser Ser Asn Ala Phe Thr Ser Gly
    130                 135                 140
Glu His Thr Asn Tyr Tyr Phe Asp Val Ser His Glu His Leu Glu Gly
145                 150                 155                 160
Ala Leu Asp Arg Phe Ala Gln Phe Phe Leu Cys Pro Leu Phe Asp Ala
                165                 170                 175
Ser Cys Lys Asp Arg Glu Val Asn Ala Val Asp Ser Glu His Glu Lys
            180                 185                 190
Asn Val Met Asn Asp Ala Trp Arg Leu Phe Gln Leu Glu Lys Ala Thr
        195                 200                 205
Gly Asn Pro Lys His Pro Phe Ser Lys Phe Gly Thr Gly Asn Lys Tyr
    210                 215                 220
Thr Leu Glu Thr Arg Pro Asn Gln Glu Gly Ile Asp Val Arg Glu Glu
225                 230                 235                 240
Leu Leu Lys Phe His Ser Thr Tyr Tyr Ser Ser Asn Leu Met Ala Ile
                245                 250                 255
Cys Val Leu Gly Arg Glu Ser Leu Asp Asp Leu Thr Asn Leu Val Val
            260                 265                 270
Lys Leu Phe Ser Glu Val Glu Asn Lys Asn Val Pro Leu Pro Glu Phe
        275                 280                 285
Pro Glu His Pro Phe Gln Glu Glu His Leu Lys Gln Leu Tyr Lys Ile
    290                 295                 300
Val Pro Ile Lys Asp Ile Arg Asn Leu Tyr Val Thr Phe Pro Ile Pro
305                 310                 315                 320
Asp Leu Gln Gln Tyr Tyr Lys Ser Asn Pro Gly His Tyr Leu Gly His
                325                 330                 335
Leu Ile Gly His Glu Gly Pro Gly Ser Leu Leu Ser Glu Leu Lys Ser
            340                 345                 350
Lys Gly Trp Val Asn Thr Leu Val Gly Gly Gln Lys Glu Gly Ala Arg
        355                 360                 365
Gly Phe Met Phe Phe Ile Ile Asn Val Asp Leu Thr Glu Glu Gly Leu
    370                 375                 380
Leu His Val Glu Asp Ile Ile Leu His Met Phe Gln Tyr Ile Gln Lys
385                 390                 395                 400
Leu Arg Ala Glu Gly Pro Gln Glu Trp Val Phe Gln Glu Cys Lys Asp
                405                 410                 415
Leu Asn Ala Val Ala Phe Arg Phe Lys Asp Lys Glu Arg Pro Arg Gly
            420                 425                 430
Tyr Thr Ser Lys Ile Ala Gly Lys Leu His Tyr Tyr Pro Leu Asn Gly
        435                 440                 445

Val Leu Thr Ala Glu Tyr Leu Leu Glu Glu Phe Arg Pro Asp Leu Ile
    450                 455                 460
Asp Met Val Leu Asp Lys Leu Arg Pro Glu Asn Val Arg Val Ala Ile
465                 470                 475                 480
Val Ser Lys Ser Phe Glu Gly Lys Thr Asp Arg Thr Glu Gln Trp Tyr
                485                 490                 495
Gly Thr Gln Tyr Lys Gln Glu Ala Ile Pro Glu Asp Val Ile Gln Lys
            500                 505                 510
Trp Gln Asn Ala Asp Leu Asn Gly Lys Phe Lys Leu Pro Thr Lys Asn
        515                 520                 525
Glu Phe Ile Pro Thr Asn Phe Glu Ile Leu Ala Leu Glu Lys Asp Ala
    530                 535                 540
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Thr Pro Tyr Pro Ala Leu Ile Lys Asp Thr Ala Met Ser Lys Leu Trp
545                 550                 555                 560
Phe Lys Gln Asp Asp Lys Phe Phe Leu Pro Lys Ala Cys Leu Asn Phe
                565                 570                 575
Glu Phe Phe Ser Pro Phe Ala Tyr Val Asp Pro Leu His Cys Asn Met
            580                 585                 590
Ala Tyr Leu Tyr Leu Glu Leu Leu Lys Asp Ser Leu Asn Glu Tyr Ala
        595                 600                 605
Tyr Ala Ala Glu Leu Ala Gly Leu Ser Tyr Asp Leu Gln Asn Thr Ile
    610                 615                 620
Tyr Gly Met Tyr Leu Ser Val Lys Gly Tyr Asn Asp Lys Gln Pro Ile
625                 630                 635                 640
Leu Leu Lys Lys Ile Thr Glu Lys Met Ala Thr Phe Glu Ile Asp Lys
                645                 650                 655
Lys Arg Phe Glu Ile Ile Lys Glu Ala Tyr Met Arg Ser Leu Asn Asn
            660                 665                 670
Phe Arg Ala Glu Gln Pro His Gln His Ala Met Tyr Tyr Leu Arg Leu
        675                 680                 685
Leu Met Thr Glu Val Ala Trp Thr Lys Asp Glu Leu Lys Glu Ala Leu
    690                 695                 700
Asp Asp Val Thr Leu Pro Arg Leu Lys Ala Phe Ile Pro Gln Leu Leu
705                 710                 715                 720
Ser Arg Leu His Ile Glu Ala Leu Leu His Gly Asn Ile Thr Lys Gln
                725                 730                 735
Ala Ala Leu Gly Val Met Gln Met Val Glu Asp Thr Leu Ile Glu His
            740                 745                 750
Ala His Thr Lys Pro Leu Leu Pro Ser Gln Leu Val Arg Tyr Arg Glu
        755                 760                 765
Val Gln Leu Pro Asp Arg Gly Trp Phe Val Tyr Gln Arg Arg Asn Glu
    770                 775                 780
Val His Asn Asn Cys Gly Ile Glu Ile Tyr Tyr Gln Thr Asp Met Gln
785                 790                 795                 800
Ser Thr Ser Glu Asn Met Phe Leu Glu Leu Phe Cys Gln Ile Ile Ser
                805                 810                 815
Glu Pro Cys Phe Asn Thr Leu Arg Thr Lys Glu Gln Leu Gly Tyr Ile
            820                 825                 830
Val Phe Ser Gly Pro Arg Arg Ala Asn Gly Ile Gln Gly Leu Arg Phe
        835                 840                 845
Ile Ile Gln Ser Glu Lys Pro Pro His Tyr Leu Glu Ser Arg Val Glu
    850                 855                 860
Ala Phe Leu Ile Thr Met Glu Lys Ala Ile Glu Asp Met Thr Glu Glu
865                 870                 875                 880
Ala Phe Gln Lys His Ile Gln Ala Leu Ala Ile Arg Arg Leu Asp Lys
                885                 890                 895
Pro Lys Lys Leu Ser Ala Glu Cys Ala Lys Tyr Trp Gly Glu Ile Ile
            900                 905                 910
Ser Gln Gln Tyr Asn Tyr Asp Arg Asp Asn Ile Glu Val Ala Tyr Leu
        915                 920                 925
Lys Thr Leu Ser Lys Asp Asp Ile Ile Lys Phe Tyr Lys Glu Met Leu
    930                 935                 940
Ala Val Asp Ala Pro Arg Arg His Lys Val Ser Val His Val Leu Ala
945                 950                 955                 960
Arg Glu Met Asp Ser Cys Pro Val Val Gly Glu Phe Pro Ser Gln Asn
                965                 970                 975

Asp Ile Asn Leu Ser Glu Ala Pro Pro Leu Pro Gln Pro Glu Val Ile
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            980                 985                 990
His Asn Met Thr Glu Phe Lys Arg Gly Leu Pro Leu Phe Pro Leu Val
        995                 1000                1005
Lys Pro His Ile Asn Phe Met Ala Ala Lys Leu
    1010                1015
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